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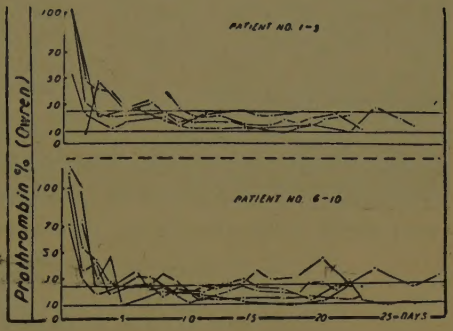

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P. A. Owren, Scand. J. Clin. Lab. Invest. **1**, 81 (1949).
P. A. Owren & K. Aas, Ibid. **3**, 201 (1951).
Chr. J. Bjerkelund, Ibid. **2**, 83 (1950).
J. Dedichen, Ibid. **5**, 324 (1953).
A. J. Hellem, Farmakoterapi nr. 2, 1953.
Rolf Jorde, Nord. Med. **50**, 1571 (1953).
Idem, Ibid. **53**, 604 (1955).

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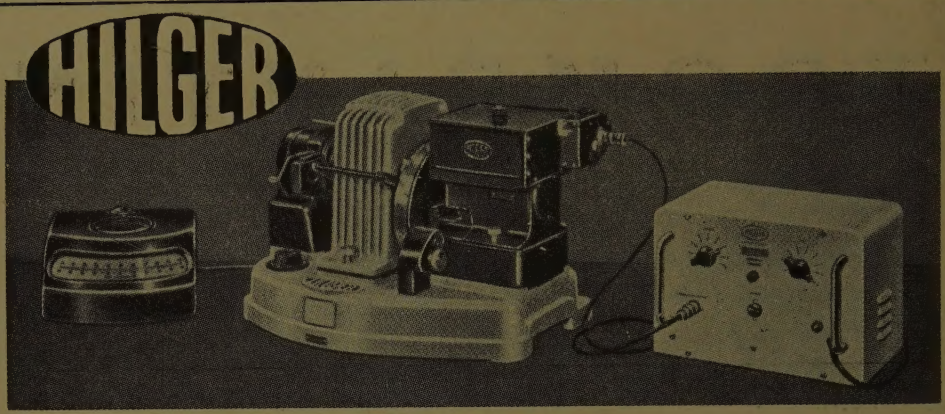

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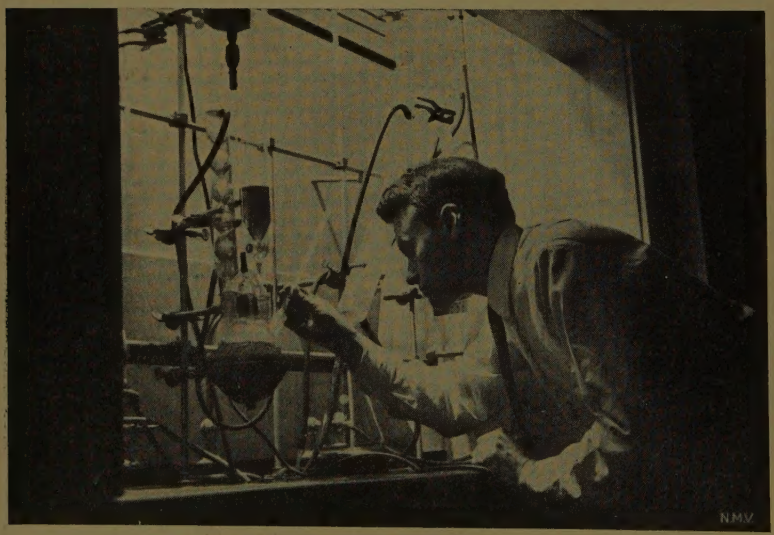

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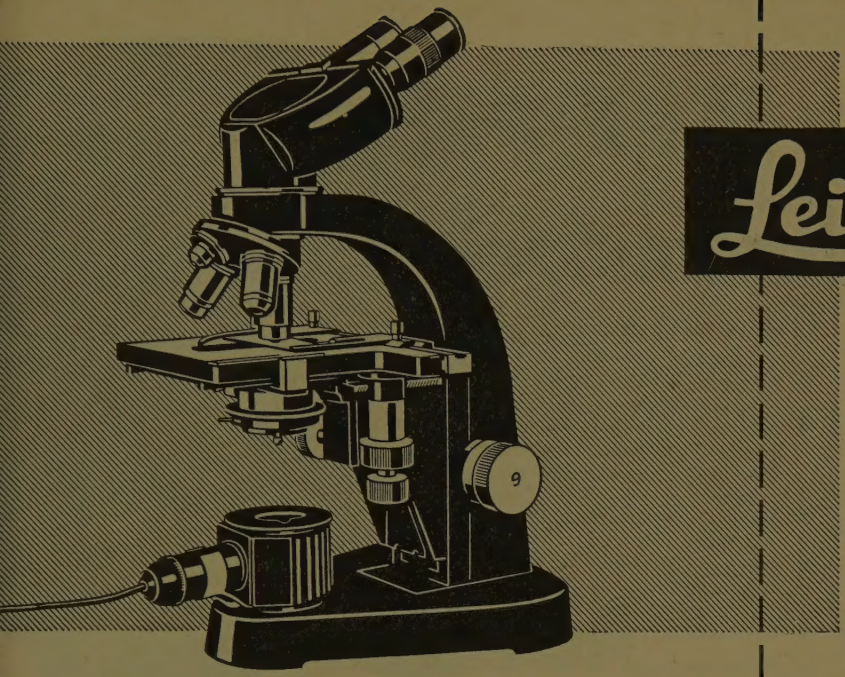

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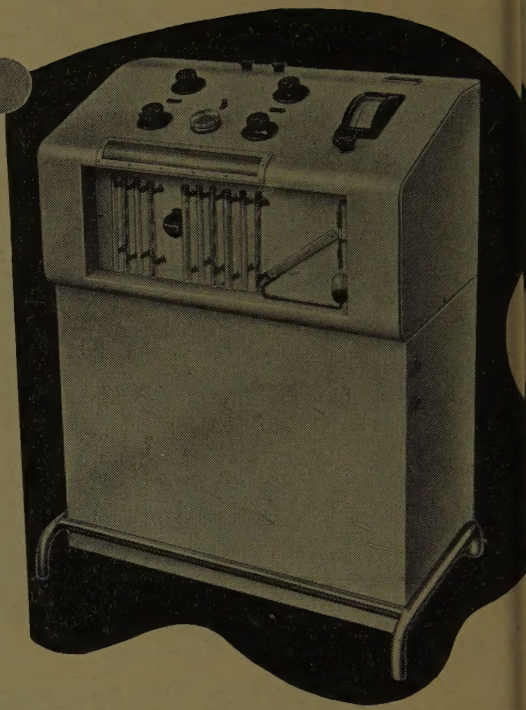

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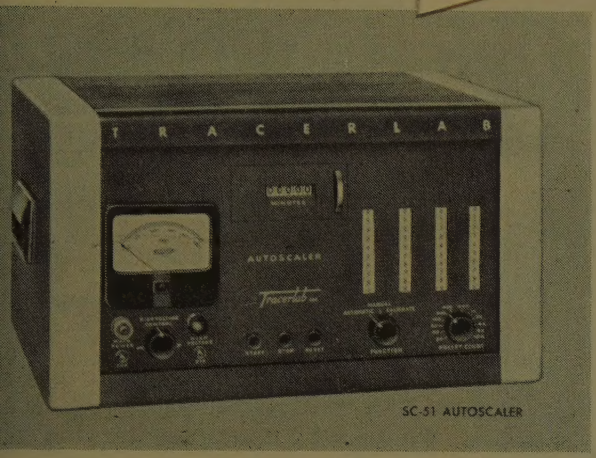

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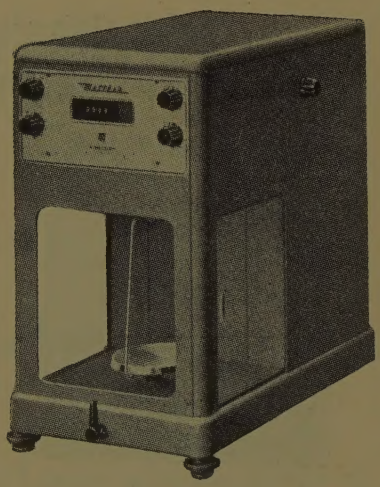

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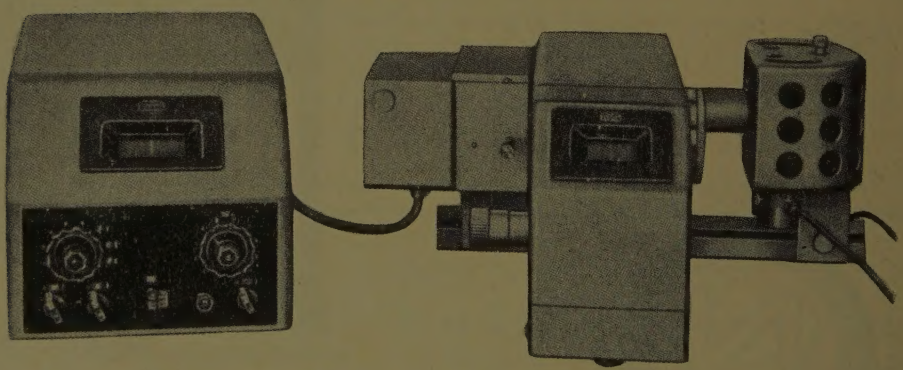

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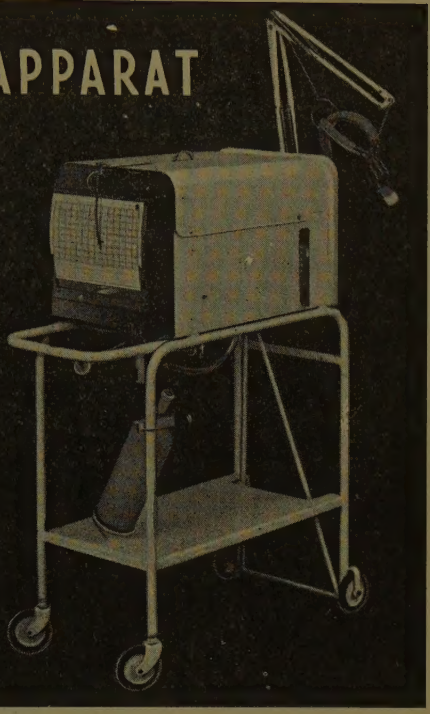

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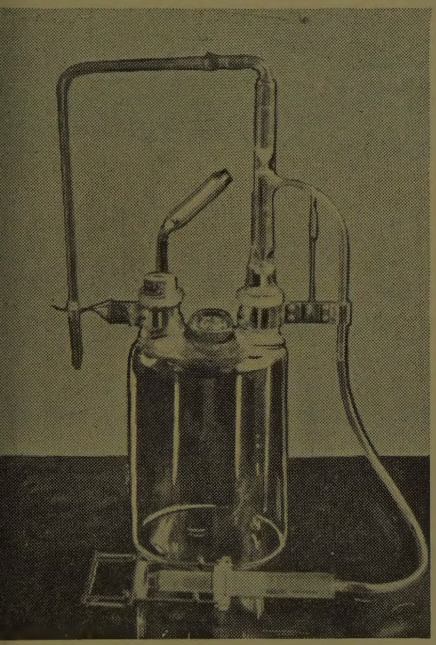

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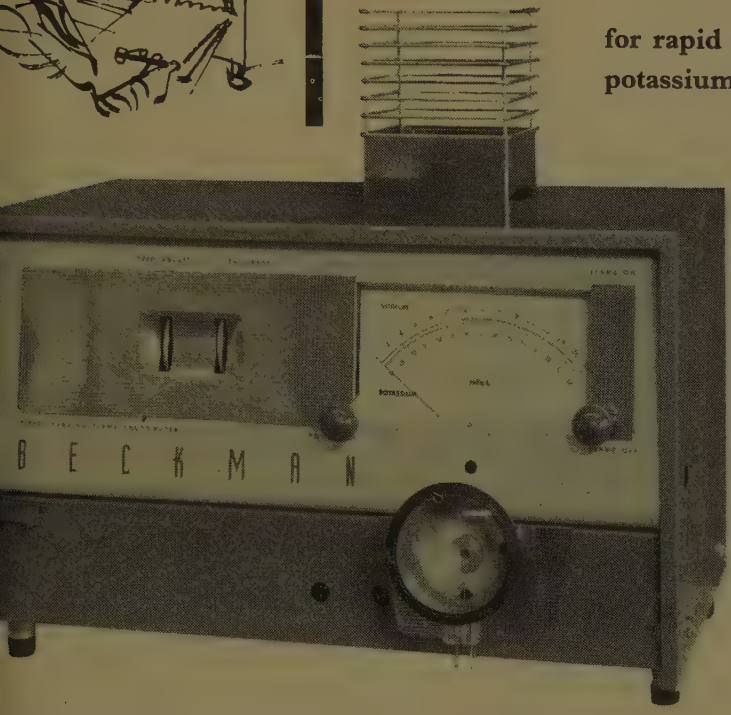

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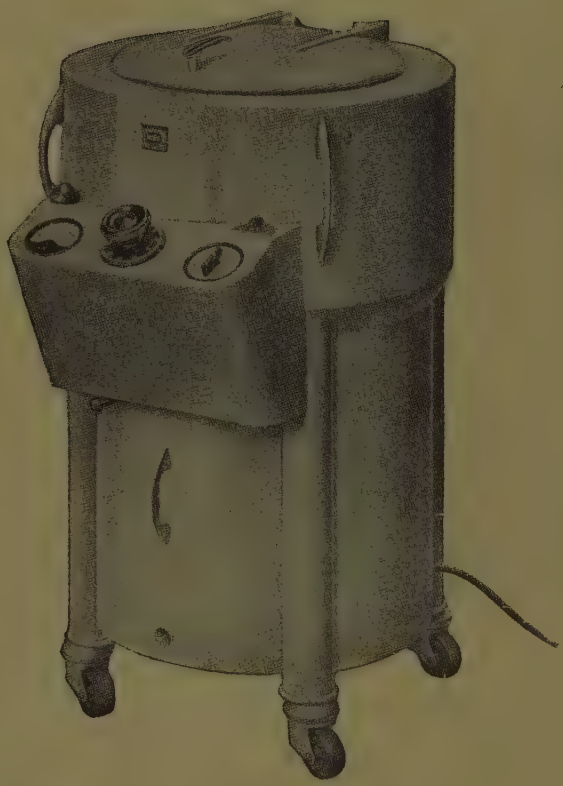

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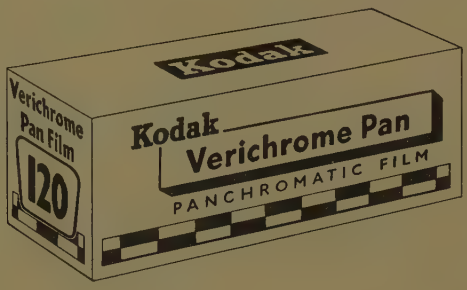

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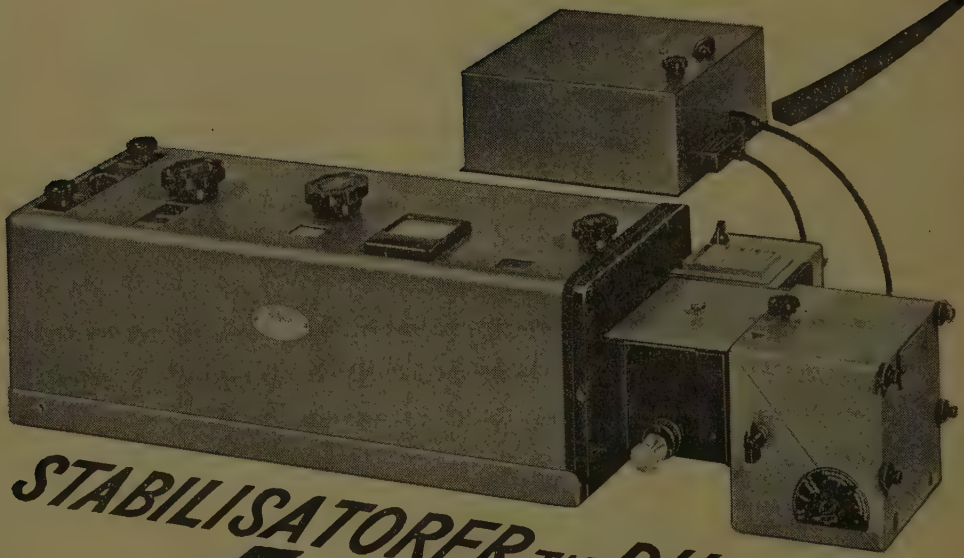

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THE NEMBUTAL METABOLIC RATE AS AN AID IN THE STUDY OF THYROID FUNCTION

By H. HORTLING and L. HIISI-BRUMMER

From Medical Department II of the Deaconess Hospital, Helsingfors, Finland

(Received for publication June 23, 1956)

espite the advances which have been e during recent years in the study of oid function in health and disease, many nostic difficulties remain. The determi- in of the serum protein — bound iodine the radio-iodine technique elucidate dif- nt phases in the hormone production of hyroid gland and hence neither of them e is conclusive; both methods are tech- ly very elaborate and the reliability of results depends on previous iodine me- ion in any form, chronic illness, *etc.* radio-iodine uptake is also altered if patient has a nontoxic goiter as is com- in endemic goiter regions (Blom, Terp- Querido, 1954; Engström, 1955; Silver, lom & Newberger, 1956; Lamberg, lberg & Kuhlbäck, 1956) such aus nd. It is also possible that the hor- e produced is normal but that the peri- al tissue cells are not able to use these ons either as thyroxine or as triiodo- onine (Reiss & Haigh, 1955; Kurland, olsky & Freedberg, 1955).

It has long been recognized that determination of the basal metabolic rate frequently gives misleading results, mainly because of its great dependence on the mental state of the patient. This method is a measure of the total oxygen consumption, which is not, however, regulated by the thyroid gland alone (Rapport, Curtis & Simcox, 1951). At any rate, determination of the metabolic rate affords a virtually pure measure of the oxygen requirement of the tissues, at least as compared with the other methods mentioned above. Some investigators have, therefore, tried to improve the reliability of the determinations of the basal metabolic rate. Attempts have mainly been concerned with elimination of the sources of error depending in different ways on psychic instability. Phenobarbital administered orally some time prior to the determination of the basal metabolic rate, *i. e.*, one night or one hour before, has not given satisfactory results. Bartels (1949) used natrium penthotal intravenously, but in some cases laryngo-

spasm developed, which needed a trained anesthetist to cure, but the method gave promising results in excluding the disturbing influence of nervous and muscular factors. Rapport *et al.* (1951) tried Nembutal, which they administered intravenously in such a way that the first minute the patient received 50 mg and thereafter 25 mg every minute until sleep supervened, whereupon the determination was performed. The average dosage was 212 mg. They claimed that such a "somnolent metabolic rate" was a better index of dysfunction in the thyroid gland than the ordinary basal metabolic rate and especially useful when nervous disturbances prevailed. In such nervous states the average difference between a determination made immediately before and after the administration of Nembutal was 32 per cent against 8.5 per cent in the other groups. Especially in cases of hypothyrosis with normal or elevated metabolic rate the somnolent metabolic rate according to Rapport may assist the diagnosis. In hyperthyrosis, too, the use of Nembutal is useful in eliminating extrathyroid components. The somnolent metabolic rate is especially helpful in situations where serum protein — iodine or the radioiodine technique are not available for different reasons but the method also has value as a measure of the total effect of the thyroid hormone on the end organs in the tissues. Others have since experimented with the same method. Walzer, Ludwig & Ortelli (1954) feel that this method does not eliminate the drawbacks of the regular determination of the basal metabolic rate. Azerad & Ravaud(1954), Leonhardt(1954) and Fraser & Nordin (1955), recommend the method.

In the IInd Medical Department of t Deaconess Hospital, Nembutal has been us for some years in connection with the dete mination of the basal metabolic rate in som what modified form compared with th method used by Rapport *et al.*.

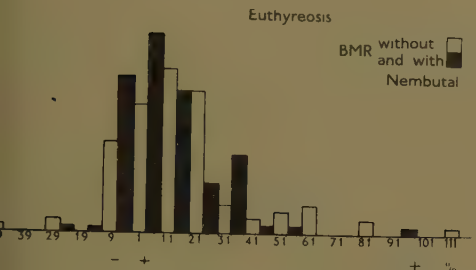
The dose of Nembutal (sodium pent barbital) used was somewhat less, *i. e.* interrupted the injection before the patie fell asleep to allow the investigation in ou patients too and the Nembutal determinatio was made either alone or the day after th ordinary determination to achieve a compa able starting condition of the patient befo the determination was made. The dose in th series was therefor less than that used b Rapport; it varied between 100—150 mg i most cases. No complications were seen bu a few patients felt somnolent some hou after the injection was made and had to re some time at the laboratory before goi home. Before the determination the patie had a rest lasting at least half an hour. Th ordinary respirometer of Krogh's type wa used.

MATERIAL

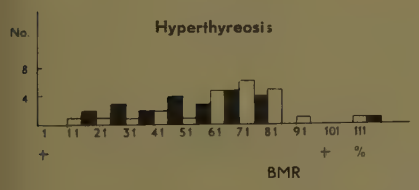
Altogether determinations of the basal metabol rate after previous Nembutal administration (NB were made on 340 patients, 173 of whom we euthyroid, 86 hypothyroid and 34 thyrotoxic a the rest undetermined. 20 determinations were ma policlinically. The material has been followed by the same observer and the final diagnosis w in most cases determined by the result of t therapy employed or a long follow-up of the patie The diagnosis of hyperthyrosis has proved som what easier than that of hypothyrosis. In the ca of hypothyrosis, the effect of administration thyroid hormone on the metabolic rate or on t patient's symptoms during at least some mont but mostly for a longer time has been evaluate

Table I. *Average results of determinations of the ordinary basal metabolic rate (BMR) and the determinations after previous Nembutal administration (NBR) in the different groups of patients.*

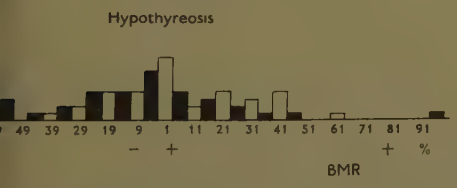
Clinical diagnosis	Number of determinations	Average results in per cent		Difference
		BMR	NBR	
Euthyrosis	93	+18	+11	7
Hyperthyrosis	25	+70	+51	19
Hypothyrosis	34	+11	— 7	18



1. Frequency distribution of the values of rminations of BMR and NBR (basal metabolic after injection of Nembutal) in the euthyroid nts. Each column indicates the number of ngle determinations of BMR and NBR in 10 per cent groups.



2. Frequency distribution of the hyperthyroid patients.



3. Frequency distribution in the hypothyroid patients.

Many of the cases of hypothyrosis are mild cases and the criteria for making this diagnosis are still a matter of controversy (Starr, 1954). As this study is only intended as a comparison of the usefulness of determinations of the basal metabolic rate with and without Nembutal, we shall not further discuss the basis for the diagnosis of hypothyrosis. At any rate all diagnoses are uniformly interpreted.

RESULTS

In 151 patients parallel determinations of NBR and ordinary basal metabolic rate (BMR) were made, the latter on the previous day. The results are seen in Table I.

An interesting point was that in the euthyroid group a comparatively large number of patients complained of neurotic symptoms. In 22 of the patients with the most typical nervous complaints, the difference between BMR and NBR was, however, the same as in the whole euthyroid group or +7 per cent.

Fig.s 1—3 show the frequency distribution of the values of determinations of BMR and NBR for the three groups of patients. Each column indicates the number of single determinations of BMR and NBR in 10 per cent groups.

In Fig. 1 are seen the determinations in the euthryroid patients. Broadly speaking the values are grouped according to the

Table II. *Comparison of BMR and NBR with respect to their usefulness in making the clinical diagnosis.*

Clinical diagnosis	Values in per cent	No. of BMR	No. of NBR
Euthyrosis	>+30	16	14*
93 cases	>+40	12	3
	<− 9	3	2
Hypothyrosis ..	>+30	8	3
34 cases	>+10	14	8
	<− 9	3	15
Hyperthyrosis	>+30	22	19
24 cases	>+10	24	24

Table III. *The usefulness of a singl determination of NBR in making the clinica diagnosis.*

Clinical diagnosis	Values in per cent	Number NBR
Euthyrosis	>+30	0
82 cases	<− 9	2
Hypothyrosis	>+30	0
53 cases	>+10	2
	<− 9	22
Hyperthyrosis	>+30	7
10 cases	>+10	10

Gauss distribution curve for normal distribution by chance, with the majority of cases grouped between — 9 per cent and + 29 per cent. These random values were also chosen as boundaries in judging the conformity of the values of BMR and NBR respectively with the clinical diagnosis.

The column of NBR values between + 31 and + 40 deserves special mention. Surprisingly the column here is much higher than the BMR column. One would think these values belonged to patients with even higher BMR, such values being seen in limited number in the graph. A closer study, however, showed that in 5 of the 11 patients in this column the NBR was higher than the BMR. Obviously the higher column to a great part is derived from a special group of patients. The number of cases in the whole series with the NBR more than 10 per cent higher than the BMR was 21. Almost all of them exhibited rather marked nervous symptoms, which were not analyzed further. The finding will be discussed later on.

Further, the histograms clearly show th overlapping of the values of the determina tions of the basal metabolic rate with an without Nembutal in the hyperthyroid an hypothyroid patients compared with th values in the euthyroid patients. Table I shows the results of a comparison of BM and NBR with respect to their usefulness i making clinical diagnosis. The random valu — 9 per cent and + 30 per cent were in co formity with the distribution of the NB and BMR values in graph 1 used to mak such a comparison possible.

Within the random values used, this tab seems to indicate that the NBR gives resul that fit the clinical diagnosis of the thyro disease better than the BMR. In the euth roid group (×) NBR was higher than BM in 7 of the 14 cases.

In order to check the validity of the figures and also the usefulness of a sing determination of NBR, NBR alone w used in another series of 143 cases. T results are reviewed in Table III.

ıe figures in Table III show fairly good
ement with the ones in Table II.
s it was possible that in general the lower
R value depended on the fact that the
rmination was made the day after the
R was measured, we made parallel in-
gations in 40 cases of NBR and BMR
everse order, that is the NBR was made
lay before the BMR. The average NBR
e was now — 0.5 per cent against + 2
cent without Nembutal. These figures
r that it is not the fact that NBR was
e after BMR that caused the difference
ıe results of the basal metabolism deter-
ıtions. They also show that NBR is at
as free from psychic influences as is
second of two ordinary determinations
ubsequent days.

s several papers have been published
ng with the limitations of the usual
ratory methods for determination of
m protein iodine, radio-iodine uptake
secretion, and serum cholesterol, we do
intend to report a detailed comparison
he determinations of the basal meta-
m with these other methods. To give
e impression of the distribution of
m protein — bound iodine in the cases
studied, the histogram in Fig. 4 was
piled. From it one is able to conclude
the values of this test vary greatly
ıe individual cases, although some gen-
trends are visible. In the group of hypo-
oid cases most values were rather low
six cases exhibited normal levels ac-
ing to the usual standards. These higher
es may depend on technical failure but
factors mentioned in the introduction
also be involved. (The determinations of
m protein-bound iodine are made in the

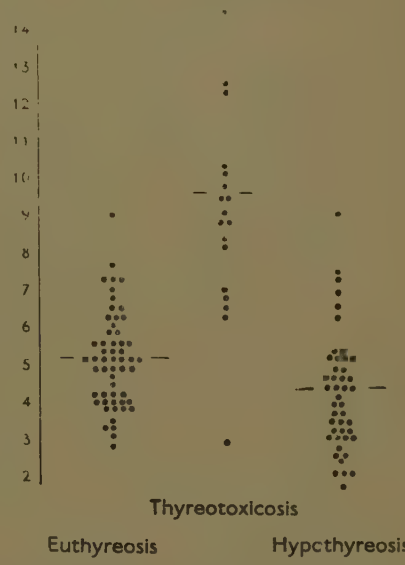


Fig. 4. Distribution of the values of the single determinations of serum protein — bound iodine in different states of thyroid function.

State Serum Institute (Prof. E. Uroma, M. D., Helsinki). Lamberg, Wahlberg & Forsius (1956) have reported results with this method. It is the intention of the authors later to discuss the diagnosis of hypofunction of the thyroid gland.

The determination of the serum cholesterol seemed to be of little value in making the diagnosis of thyroid dysfunction, perhaps with the exception of hyperthyroid states. In 118 cases of euthyroidism studied, the average serum cholesterol was 254.4 mg/100 ml, in 75 hypothyroid patients the average was 275 mg/100 ml and finally in 31 hyperthyroid patients 179.2 mg/100 ml. There was in all groups a wide dispersion of the values.

DISCUSSION

This study indicates that the determination of basal metabolic rate after a previous intravenous injection of Nembutal is more useful than the ordinary determination of the metabolic rate in making a diagnosis of the functional state of the thyroid gland. However, the study also showed that frequently the basal metabolic rate after administration of Nembutal gives misleading values. The present data are insufficient to allow a comparison of the usefulness of the basal metabolic rate with that of the other diagnostic aids such as determination of the serum protein iodine and the radio-iodine technique. Obviously the clinical picture and the result of the specific therapy in clinically disturbed thyroid function are at all times the most reliable guides in making the diagnosis of such patients.

The observation of NBR values more than 10 per cent higher than the previous BMR in 21 cases deserves some mention. Rapport *et al.* also observed this phenomenon in their material but were not able to offer any explanation. In our cases mental disturbances dominated the clinical picture. Since Rapport's study, Schwarz (1955) and Schwarz, Kinderen & van Haalten (1955) in animal experiments have demonstrated an increase in the uptake of radioactive iodine and serum protein — iodine (PPI^{131}) after administration of prominal to rats. They asked for similar observations in clinical patients. It is perhaps possible that the higher NBR in this material may be a concequence of a release by Nembutal of a central inhibition of the thyroid function. Such an inhibition has not, as far as we know, been described in clinical material and perhaps deserves some attention in the future.

It is difficult to know exactly how Nembutal administration changes the basal metabolic rate. Apparently the most important effect is to exclude some central nervous agitation that makes the values more labile. Very careful technical skill in carrying out the ordinary basal metabolic estimation may probable lessen, at least to some degree, the disturbing effect of a labile mental state even without the use of Nembutal as was observed in this material too during the course of the investigation, but it seems that Nembutal makes the basal metabolic rate more reliable in general clinical practice.

SUMMARY

In 293 patients where the diagnosis had been made on the basis of thorough observation and the results of therapy, determination of the basal metabolic rate was made after previous intravenous administration of Nembutal in a dosage averaging 100–150 mg. 86 patients were hypothyroid, 34 hyperthyroid and 173 euthyroid.

In 151 patients an ordinary determination of the basal metabolic rate (BMR) was made the day before the Nembutal basal metabolic rate (NBR). In the euthyroid group the mean difference between BMR and NBR was 7 per cent (+ 18 per cent — + 11 per cent), in the hyperthyroid patients (25) the difference was greater (+70 per cent — +5 per cent or 19 per cent) and in the 34 patients with hypothyrosis the difference was 18 per cent (+ 11 per cent — — 7 per cent). In the euthyroid patients the same difference, 7 per cent, was seen in 22 selected patients with obvious symptoms of nervous instability.

the patients with hyperthyrosis, both
and NBR were elevated in almost all
but in the hypothyroid and euthyroid
its the NBR fitted the clinical diag-
better. In 171 cases only the NBR was
ired and the result as compared with
inical diagnosis was about the same as
an ordinary BMR was measured the
efore.

21 cases with predominantly nervous
toms, the NBR was more than 10 per
higher than the BMR. The possibility
central nervous inhibition in these cases
liscussed.

the diagnosis of the individual case of
yrosis the NBR must, after all, be re-
d as a rather unreliable method, per-
with the exception of hyperthyroid
its, but a comparison with the results
rum protein — iodine determinations
serum cholesterol values showed the
to be true with these methods too.

REFERENCES

l, E. & Ravaud, C.: Valeur respective
métabolisme de base et de l'épreuve de fixa-
on de l'iodie radioactif dans l'exploration
nctionelle de la glande thyroide. Semaine hôp.
aris. *30,* 1373, 1954.

s, E. G.: Basal metabolism testing under
ntothal anesthesia. J. Clin. Endocrinol. *9,*
90, 1949.

P. S., Terpstra, J. & Querido, A.: Storende
vloeden bij het schildklier-onderzoek met be-
lp van radio-actif jodium. Nederl. Tijdschr.
eneesk. *98,* 842, 1954.

orn, O.: Die Problematik des Schilddrüsen-
nktionsprüfungen mit Radiojod in Jod-
angelgebiete. Schweiz. med. Wchnschr. *85,*
9, 1955.

öm, W. W. & Markardt, B.: The effects of
rious illness and surgical stress on the cir-
culating thyroid hormone. J. Clin. Endocrinol. *15,* 953, 1955.

Fraser, R. & Nordin, B. E. C.: Basal metabolic rate during sleep. Lancet *1,* 532, 1955.

Kurland, G. S., Hamolsky, M. W. & Freedberg, A. S.: Nonmyxedematous hypometabolism: The clinical syndrome and the effects of triiodethyronine, alone or combined with thyroxine. J. Clin. Endocrinol. *15,* 1354, 1955.

Lamberg, B. A., Wahlberg, P. & Forsius, P. I.: The serum protein-bound iodine as a dignostic aid. Acta med. Scandinav. *154,* 201, 1956.

Lamberg, B. A., Wahlberg, P. & Kohlbäck, B.: I^{131}-excretion vid endemisk struma i Finland. Nord. Med. *55,* 354, 1956.

Leonhardt, K.: Die Differentialdiagnose von Schilddrüsenkrankheiten und vegetativen Störungen mit Hilfe der Grundumsatzbestimmung im Schlaf. Münch. med. Wchnschr. *100,* 284, 1953.

Rapport, R. L., Curtis, G. M. & Simcox, S. J.: Somnolent metabolic rate in diagnosis of thyroid dysfunction. J. Clin. Endocrinol. *11,* 1, 1951.

Reiss, M. & Haigh, C. P.: Various forms of hypothyroidism in mental disorders. Proc. Roy. Soc. Med. *47,* 889, 1954.

Schwarz, F.: The influence of prominal on the I^{131} uptake of the thyroid gland of the rat. Acta Endocrinol. *18,* 33, 1955.

Schwarz, F., Kinderen, P. J., van Haalten, J. L. & Koopman, L. E.: Further investigations on the effect of prominal on thyroid function of the rat. Acta Endocrinol. *19,* 411, 1955.

Silver, S., Yokalim, S. B. & Newberger, R. A.: Pitfalls in diagnostic use of radioactive iodine. J. A. M. A. *159,* 1, 1955.

Starr, P.: Hypothyroidism. Springfield, 1954.

Walzer, H., Ludwig, H. & v. Ortelli, G.: Der diagnostische Wert der Bestimmung des Serumproteinjods und des Schlafgrundumsatzes. Helv. med. Acta *21,* 1549, 1954.

Zieve, L., Skanse, B. & Schultz, A.: Comparative value of the basal metabolic rate, chemical protein-bound iodine, and radioactive iodine excretion or uptake in the diagnosis of borderline hypothyroidism when used individually or in combination. J. Lab. Clin. Med. *45,* 281, 1955.

Scandinav. J. Clin. & Lab. Investigation 8—14, 9, 1957.

A SIMPLE TWO-CHAMBER ELECTRODIALYZER

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In dealing with proteins it is often desired to remove low-molecular weight electrolytes as completely and as rapidly as possible. This is usually effected by electrodialysis, which is far superior to ordinary dialysis when the specific conductance of the solution is lower than 10^{-3} mho cm^{-1}, a field strength of sufficient magnitude being applicable without the development of too much heat.

For details the interested reader is referred to a very able review by R. E. Stauffer (1950) on the theoretical and practical aspects of dialysis and electrodialysis. He describes a number of laboratory electrodialyzers, some of which are rather complicated, but states that the simplest design consists of three chambers separated by semipermeable membranes.

Effective electrodialysis may be performed, however, by an even simpler apparatus of the two-chamber design to be described below. It consists simply of a dialyzing bag suspended between two electrodes in a vessel containing continuously renewed water.

APPARATUS

Fig. 1 illustrates the apparatus the details of which are given in the legend. This arrangement of the dialyzing bag was used in all the experiments, the glass rod in its centre being neces as an axis to secure regular rotatory motion (*infra*). By means of the glass tube at the to the bag, samples could be drawn for conductiv measurements. In routine work no special eq ment is required.

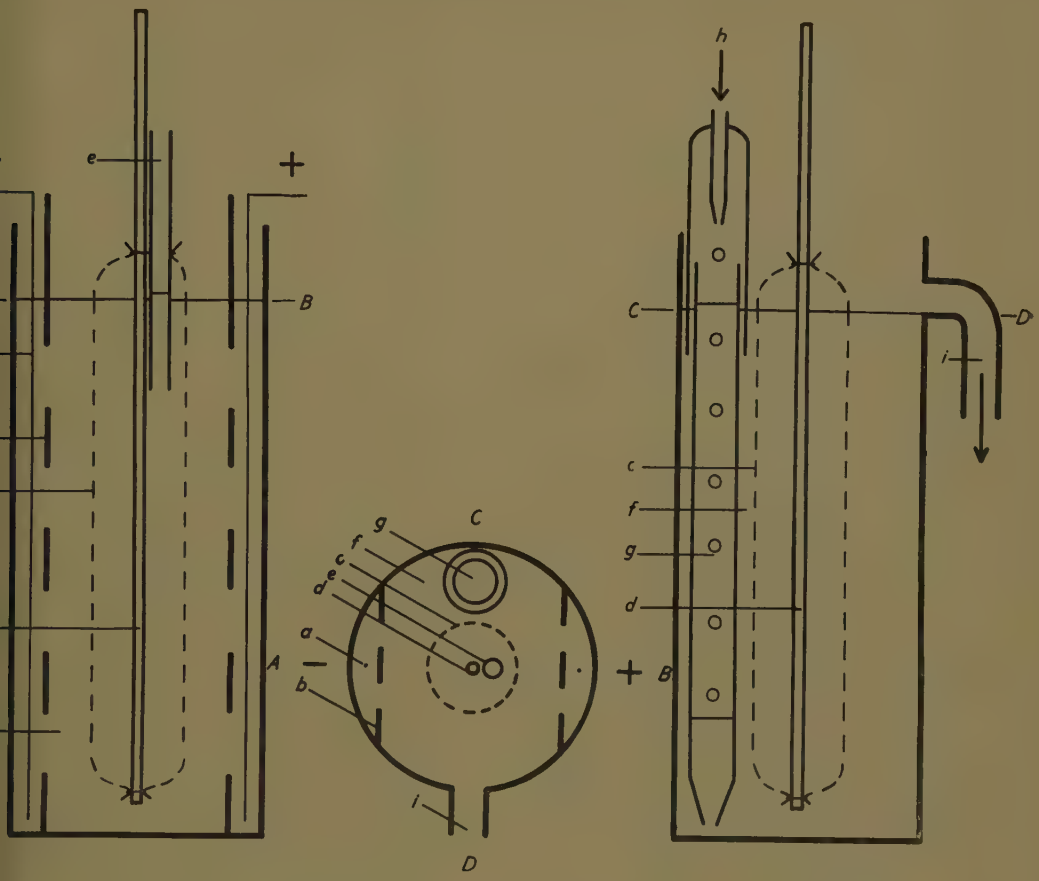
Platinum electrodes 0.30 mm in diameter 200 mm in length, are mounted on Plexiglas pl in a way which prevents contact between the and the electrodes.

Distilled water (specific conductance 0.7 t $\times 10^{-6}$ mho cm^{-1}) is passed through a t mounted in a cap which *in situ* encases the up part of a wide glass tube almost filled with tolu This involves the advantages of (1) bacteriosta as by falling through the toluene the water beco quite well saturated with it (2) visual control the water flow, (3) toluene and water conta toluene are not in contact with rubber tubi (4) little toluene odor in the laboratory, and toluene may be renewed without any difficulty just lifting the cap and filling the tube.

Distilled water is conveyed to the bottom of dialyzing vessel, while the outflow is from uppermost layer of water. This gives a tende to displacement of the warm water containing e trolytes, as its density is lower than that of cold water which has just entered. The electro concentration in the warm water is so slight its effect upon the density may be left out account.

The outlet from the vessel must, in its proxi part, have a lumen of at least 10 mm to a blocking by air bubbles which may lead to o flowing.

* The expenses of this investigation were defrayed by a grant from The Danish State Research Foundation.



1. Electrodialyzer. Dimensions: Radius 51 mm. Capacity: 1400 ml. *a* platinum electrodes, *b* elec-
e screening, *c* dialyzing bag, *d* glass rod, rotation axis of the bag, *e* glass tube to remove samples,
f distilled water, *g* toluene, *h* inlet of distilled water, *i* outlet.

EXPERIMENTAL

e experiments were carried out at room
rature. In the electric circuit the dialyzer and
stance of 1000 ohms were joined in series,
g the maximum effect in the vessel 12 and
tts at a fall in the voltage of 220 and 440
respectively throughout the apparatus. The
rature in the uppermost and warmest layer
ter was controlled during all the experiments,
decides the minimum water flow. Temper-
exceeding 42° C did not occur.

In all the experiments Visking Cellulose Sausage Casings were used as dialyzing bags. Where nothing else is stated, the bags had a radius of 8.5 mm.

All the conductivity measurements were performed at 0.00° C at a LKB conductivity bridge.

RESULTS AND DISCUSSION

Fig. 2 exemplifies the results of an electrodialysis experiment with 20 ml of 0.01 m KCl in the bag and Table I gives an impression of the dialysis rate for various electro-

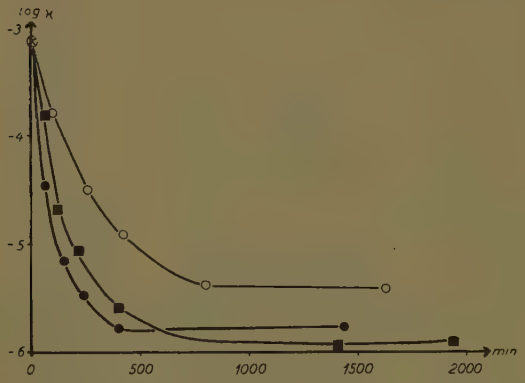


Fig. 2. Electrodialysis of 20 ml 0.01 m KCl. Abscissa: Time in minutes, ordinate: logarithm of specific conductance of the solution. Symbols: O no field applied, no rotation of the bag, ● 440 volts, without rotation, ■ 440 volts, 0.47 r.p.m. Starting water flow about 450 ml/h, gradually falling to about 200 ml/h.

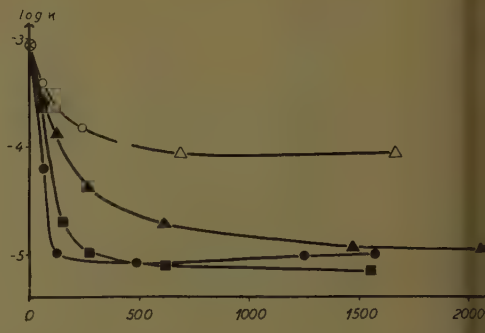


Fig. 3. Electrodialysis of 20 ml 4.1 g per c ovalbumin in 0.01 m KCl, starting pH 5.5. Coor nates as in Fig. 2. Symbols: O without electr field, without rotation, △ without electric fi 32 r.p.m., ● 440 volts, without rotation, ■ 4 volts, 0.47 r.p.m., ▲ 440 volts, 32 r.p.m. Starti water flow about 400 ml/h, gradually falling the course of about 1500 minutes to about 200 ml

lyte solutions. As might be expected in view of the negatively charged membrane, dialysis is slower using K_2HPO_4. The experiments using protein solutions are illustrated in Figs. 3 and 4 and explanations given in the legends.

These experiments show that the apparatus acts as an effective electrodialyzer. In addition to its simplicity and low cost, the method has other advantages above the ordinary apparatuses. For instance, it is well-suited for handling small samples and it makes it possible to run a salt-precipitated protein or a solution with high electrolyte concentration down to a practically salt-free solution without having to change the dialyzing bag (reduced risk of bacterial contamination), involving only the increase in volume conditioned by the dilatation of the dialyzing bag which is tied at both ends. In practice,

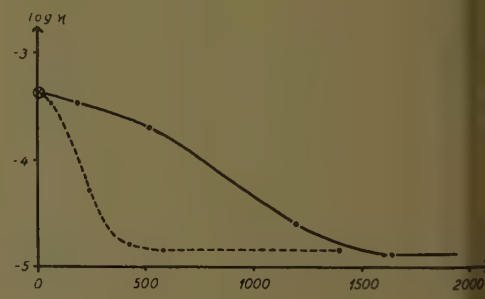


Fig. 4. Electrodialysis of 12.6 g per cent ovalbum in 0.008 m NaCl, pH 5.4 440 volts, no rotati Dotted curve for 20 ml solution in dialyzing b with a radius of 8.5 mm, water flow 400 → 3 ml/h. Solid line for 150 ml solution in bag with radius of 21 mm, water flow 1000 → 200 ml/h.

the electrodialysis is completed when t electric current is nearly identical with t current in the apparatus before the dialyzi bag was suspended.

ther advantage is that it involves the
ility of using a rotating dialyzing bag.
bjects is partly to secure stirring, but
rily to make the direction of the elec-
urrent through the walls of the dia-
bag alternating. Bethe & Toropoff
ter Manegold & Kalauch have studied
ndency of any membrane placed in a
n between electrodes to assume a
d condition on the two faces of the
ranes such that the surface nearest the
becomes negatively charged and the
earest the cathode positively charged.
results in an accumulation of hydroxyl
(alkaline reaction) at the anode face,
n acid reaction at the cathode face.
ally, these phenomena are counteracted
ing a positively charged anode mem-
(anion permeable) and a negatively
d cathode membrane (cation perme-
It is no uncommon finding in electro-
is, however, that deposits of amor-
, at times even more or less denatured
n accumulate on the membranes.
use of a rotating dialyzing bag, which
ates the direction of the electric cur-
hrough the membrane, might be im-
l to obviate or reduce shifts in local
ns and/or protein accumulations. Of
, the electrodialysis is slower this way
vith direct current, but Tables I and
well as Figs. 2 and 3 show that dialysis
ds at an acceptable rate and that the
te values for conductance are close to
obtained with direct current without
n. It is amusing to note that all ulti-
values obtained at 0.47 rotations per
e are even below those found with
current alone (except in experiments
$AlCl_3$, in which the ultimate values are
al).

Table II summarizes some experiments using alkali-denatured β-lactoglobulin which has a marked tendency to form amorphous deposits on the walls of the dialyzing bag during electrodialysis. It will be seen that by employing rotation it is possible to obtain lower values for conductance before deposit formation sets in. The values given for the dialysis experiments (0 volts) may be reduced further, but there is only a slight tendency to a further decrease.

Other experiments showed that regular alternating current without rotating bag, but with a frequency corresponding to the rotation experiments, gives poorer results, both as regards the dialysis rate and protein deposit formation.

Fig. 5 sets out the result of an electrodialysis experiment (220 volts, no rotation) with serum from a patient with hepato-

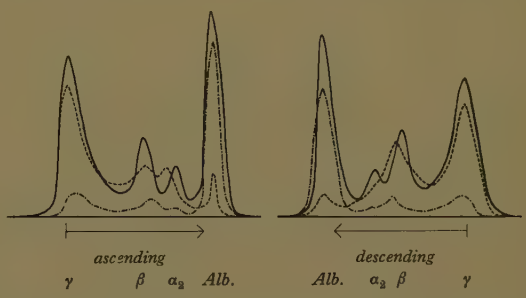


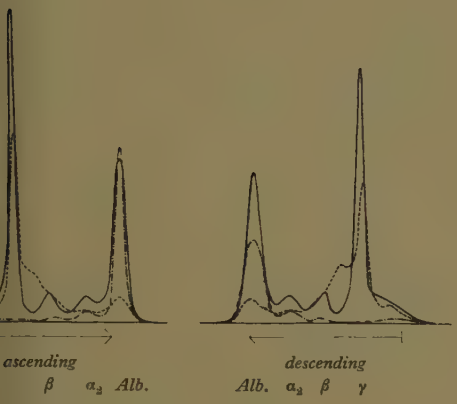
Fig. 5. Electrophoretic diagrams (moving boundary method). Serum from patient with hepatomegaly, γ-protein of normal "antibody" type. ———— untreated serum, - - - - - - - euglobulins prepared by means of electrodialysis (220 volts, no rotation), —·—·— proteins still in solution after electrodialysis. The last two curves are plotted so that the sum of their areas is equal to the area below the curve representing untreated serum, whereas the mutual relation of their areas is equal to the relation between the amount of precipitated protein and protein still in solution.

Table I. *Dialysis and electrodialysis of saline solutions. Starting water flow 450–400 ml/h, gradually falling to about 250 ml/h at the end of 1000 minutes.*

Starting solution 20 ml solution	Time in minutes for dialysis from specific conductance 10^{-4} to 10^{-5} mho cm^{-1}.		
	0 volts No rotation	440 volts 0.47 rot./min.	440 volts No rotation
0.003 M $AlCl_3$ average temp.	240 23°C	85 30°C	60 32°C
0.01 M KCl average temp.	340 26°C	135 36°C	75 33°C
0.003 M K_2HPO_4 average temp.	1050 21°C	185 29°C	60 30°C

Table II. *Dialysis and electrodialysis of 4.9 g per cent alkali- denatured β-lactoglobulin in 0.2 M NaCl, pH 9.2. Starting solution in dialyzing bag 15 ml. Starting water flow 500 ml/h, decreasing to about 200 ml/h at the end of 3000 minutes. In the experiment using 440 volts, however, the flow had to be increased to 700 ml/h in order to keep the temperature below 41° C.*

Volts	Rot. per min.	Min. after start	Specific conduct. × 10^6	pH	Appearance of	
					solution	membrane
0	0	3020	207	7.3	clear	no deposits
0	0.47	2480	208	7.25	»	»
0	32	2775	153	6.6	»	»
220	32	1810	139		»	»
		2795	66		slightly turbid	»
		3240	39		very turbid	»
		4275	19		opaque	»
		4695	16	5.5	incipient precipitation	thin deposit
440	32	60	757		clear	no deposits
		210	110		slightly turbid	deposits in spots
220	0.47	90	1473		clear	»
		180	588		»	marked deposit formation
		330	145		bag filled with deposits.	
220	0	90	1419		membrane towards anode entirely covered with deposits	
		180	218		bag filled with deposits	
		1280	1.2	5.0	bag filled with deposits in flakes and firm lumps.	



6. Electrophoretic diagrams. Serum from
t with macroglobulinaemia, γ-protein of nor-
s well as abnormal type. Plotted like Fig. 5.
Electrodialysis: 220 volts, no rotation.

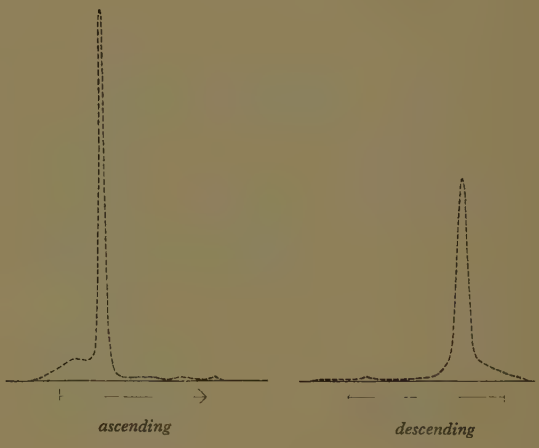


Fig. 7. Electrophoretic diagrams. Euglobulin prepared by means of electrodialysis. 220 volts, 32 rotations per minute.

ly. As much as 60 per cent of the protein
precipitated as amorphous masses
obulins). The figure shows electro-
etic diagrams of the original serum pro-
euglobulin, and the fraction soluble in
ree solution (pH 6.5). It is evident that
2 protein is not left unchanged by the
dure.

g. 6 presents a similar experiment with
n from a patient with macroglobuline-
(Waldenström). Only a small propor-
of the euglobulin was precipitated in
phous, flakes, the greater part forming
ar jelly of a consistency like a ripe
gage. The appearance bore a striking
blance to cryoglobulin, but despite
ng the saline concentration, it was not
ble to make the jelly dissolve at about
and again separate into two layers in
old. Part of the abnormal protein has
altered in the course of electrodialysis.

In contrast to this Fig. 7 presents the euglobulin produced by 220 volts and 32 rotations per minute. Here is not possible to detect any modifications.

SUMMARY

A very simple electrodialyzer is described and its effectiveness illustrated. In its present design this apparatus is well-suited for handling volumes of 150 ml and less, and it also makes it possible to carry through dialysis from salt-precipitated protein to salt-free protein solution in the same closed dialyzing bag. By this means, the risk of infection is reduced, and the volume of the sample is only increased by the amount which corresponds to the dilatation of the bag. Lastly, by rotating the bag, alternating current may be passed through the dialyzing membrane, reducing the significance of the membrane phenomena which threaten the stability of many proteins.

REFERENCES

Bethe, A. & Toropoff, T.: Über elektrolytische Vorgänge an Diaphragmen. Teil I. Die Neutralitätsstörung. Ztschr. f. physik. Chemie *88*, 686, 1914.

— — Über elektrolytische Vorgänge an Diaphragmen. Teil II. Die Abhängigkeit der Grösse und Richtung der Konzentrationsänderungen und der Wasserbewegung von der H-Ionenkonzentration. Ztschr. f. physik. Chemie *89*, 597, 1915.

Manegold, E. & Kalauch, K.: Über Kapillarsyst me. Die Neutralitätsstörungen an stromdurc flossenen Membranen. Kolloid-Ztschr. *86*, 3 1939.

Stauffer, R. E.: Dialysis and Electrodialysis. Weissberger: Technique of Organic Chemistr Vol. III. Interscience Publ., Inc., New Yor 1950, pp. 313—361.

linav. J. Clin. & Lab. Investigation 15—20, 9, 1957.

COMPARISON BETWEEN TWO DIFFUSION METHODS FOR DETERMINATION OF BACTERIAL SENSITIVITY TO ANTIBIOTIC AGENTS

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recently published method of determin- bacterial sensitivity to antibiotic and iotherapeutic agents (Czerkinsky, Diding Ouchterlony, 1955) has been compared an earlier method of analysis of bac- l sensitivity (Ericsson, Högman & kman, 1954). In both methods filter r impregnated with antibiotics is placed ie surface of an agar plate and the anti- cs diffuse from the paper into the um. In the method described by Czer- ky, Diding & Ouchterlony, strips im- nated with antibiotic and chemothera- c agents are used. Disks, 5 mm in dia- r, made of filter paper are used by sson in his method.

e strips obtained from ACO (Apotekar- etens Laboratorieavdelning, Stockholm) prepared by a special machine so that ntibiotic agents appear as equally-spaced rent colored rectangles. They are in the wing sequence reading from the black at one end of each strip: sulfathiazole, illin, chlortetracycline (aureomycin), tracycline, (terramycin), chloramphe- (chloromycetin) and streptomycin. The vening spaces are printed with linseed o prevent the agents from diffusing n the strip. Disks impregnated with the same agents as above were obtained from Bakteriologiska Laboratoriet, Karolinska sjukhuset, Stockholm 60.

The purpose of this investigation was to compare the accuracy of these two methods by analysis of the "error of the method" and the correlation coefficient.

MATERIAL

Bacteria

Two hundred eighty bacterial strains were isolated from urinary and respiratory tract specimens from clinical patients including gram negative and gram positive rods and cocci.

The strains cover all four sensitivity groups proposed by Ericsson, as can be seen in Fig. 3. The test material can therefore be considered as representative of the bacterial strains usually handled by a routine laboratory for sensitivity determination.

Staphylococcus aureus 200-P was used as a reference or standard strain.

TECHNIQUE

Medium

Peptone-free Blood Agar:

Agar — 20 grams
Placenta-infusion — 1000 ml
NaCl — 3 grams
Dextrose — 0.5 gram
Dinatrium phosphate — 1.0 gram
Horse blood — 5 per cent of total volume.

Disk test

Ericsson's method has been described elsewhere in detail (Ericsson *et al.*, 1954) and only a brief review of the technique used will be given. Samplings from 5—10 colonies of the strains to be tested were suspended in 2.5 ml saline. One drop of the suspension was placed with a pipette on each of two peptone-free blood agar plates (10 cm diameter) and spread over the surface with a triangle shaped platinum wire. Three filter paper disks, impregnated with different antibiotics, were placed on each plate. The plates were left at room temperature for three hours and then incubated for 15—18 hours at 37° C. The greatest diameter of the zone of inhibition was measured with calipers and recorded in mm.

Strip test

Strip application

The impregnated strip was placed with a sterile forceps across the middle of a peptone-free blood agar plate (10 cm). The ends of the strip were pressed into the agar surface since they have a tendency to curl from the agar. Should this occur the readings for sulfathiazole and streptomycin would be misleading.

Inoculation

To ensure a representative suspension, samplings were taken from 5—10 colonies of a test strain and suspended in 2.5 ml saline. After a suspension of minimum turbidity was prepared, one drop from a pipette was placed on one side of the plate and spread evenly with a platinum triangle up to the strip. Care was taken to avoid moistening the strip with the inoculum. A similar suspension of the standard strain was used to inoculate the other half of the plate. The ideal inoculum should give an even growth of non-confluent colonies. Too heavy a growth will result if the bacterial suspension is too dense or the inoculum too large.

Incubation

The plates were left at room temperature for three hours and then incubated for 15—18 hours at 37° C.

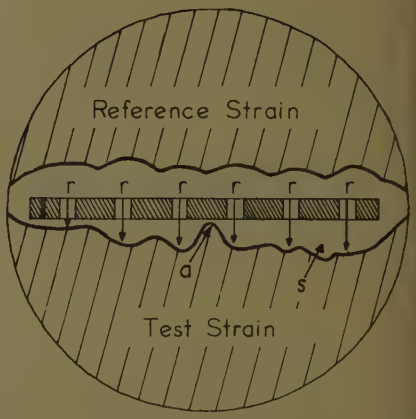

Fig. 1. Test strip with inhibition zones on ag plate. — The arrows a and s indicate antagonis and synergism between two antibiotics. The arro r show the distances measured for readings.

Readings

The zone of inhibition was measured with c lipers and recorded in mm. The greatest distan on a line perpendicular to the strip through t antibiotic rectangle was taken. The width of t strip (5 mm) was included to facilitate the re ings (Fig. 1).

Control

The standard strain was used as a control of t medium, the strip and each antibiotic agent. If a abnormality occurred, *e. g.* unusable medium strip, or a strip separated from the agar, the rea ing for that strip or single antibiotic was exclud

CALCULATIONS

Since inoculation, incubation and readin were performed the same way in the tw methods we may compare directly their e ficacy in determining bacterial sensitivit This was done by calculating for each an biotic agent used in each method the "err of method" and the correlation coefficien

ERROR OF METHOD

measures of the standard strain from the s of each method were chosen at random ir mean values and standard deviations were ted (Table I). These figures were used pute the errors of the method in the follow- rmula (Croxton, 1953):

$$V = \frac{\sigma}{M} \cdot 100$$

summary of these figures shows that ean error for the strip method is 9.3 ne range between the highest and the values is 4.9. The corresponding val- or the disk method are: mean 8.2 and 4.0. Excluding the results for chlor- enicol in the strip figures, a mean of d a range of 4.1 are found.

CORRELATION COEFFICIENT

order to compare the disk method read- o those of the strip method, it was ne- y to convert the latter readings to eter measures". The conversion for- used was 2a—5 = diameter measure , in which "a" equals the strip method g in mm. This diameter measure will ximate the reading of an inhibition which would surround a single filter rectangle placed within an inoculated

ording to Erlansson (1951) the diffu- radient of an agent, applied as de- d, may for practical purposes be con- d a straight line if the concentrations otted in logarithmic scale and the dis- from the diffusion center in linear The gradients for different concen- s of the same agent will be parallel. oncentration of the antibiotic agent at riphery of the inhibition zone for the same strain may be considered to be the same. It is therefore possible to calculate the linear distance between two gradients for the same agent by determining one point on each gradient. With this figure one may adjust the measurements of inhibition found by two different methods, one to the other, even if the initial concentrations varied (Fig. 2). These considerations were used in the calculation of the "disk unit conversion term" (Table I).

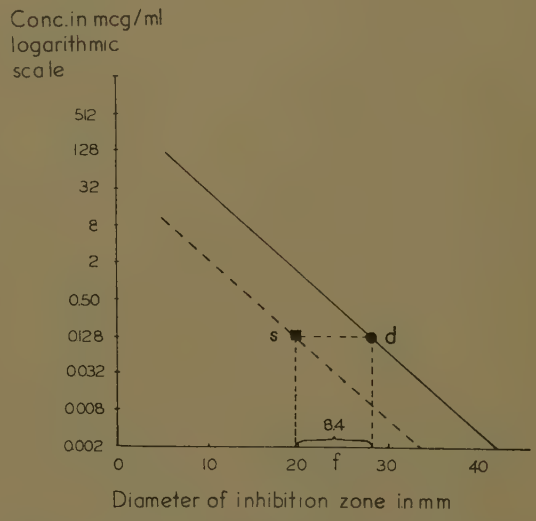

Fig. 2. Method of calculating Disk Unit Conversion Term. — In this example Ericsson's diffusion gradient for oxytetracycline is plotted and the experimental mean disk measure established on the gradient (d). The mean diameter measure for the strip method is placed at the same dilution (s), and a line parallel to the disk gradient drawn through the strip point. The linear distance between the two gradients or the "disk unit conversion term" (f) is thus obtained by subtracting.

The strip method diameter measures were converted to "disk units" by adding the "disk unit conversion terms" (Table I). Since

Table I. *Strip method and disk method. Determination of standard deviations, erro of method and disk unit conversion terms, using readings for the standard strain (Staph. aureus 209-P).*

		Sulfa drug	Penicillin	Chlortetracycline (Aureomycin)	Oxytetracycline (Terramycin)	Chloramphenicol (Chloromycetin)	Streptomycin
Strip	Concentration	0.1 mg	1.0 I.U.	10 γ	20 γ	20 γ	50 γ
	Mean actual measurement of inhibition in mm	13.52	14.12	12.84	12.41	11.62	13.42
	Standard deviation of actual measures	1.3	1.0	1.0	1.0	1.4	1.5
	Error of method	9.6	7.1	7.8	8.1	12.0	11.2
Disk	Concentration	2.4 mg	20.0 I.U.	50 γ	50 γ	30 γ	50 γ
	Mean diameter measure in mm	30.5	33.0	21.7	28.2	23.0	24.0
	Standard deviation of diameter measures	2.7	3.3	1.8	1.7	1.8	1.8
	Error of method	8.9	10.0	8.3	6.0	7.8	7.5
Strip	Mean actual measurement of inhibition in mm (a)	13.52	14.12	12.84	12.41	11.62	13.42
Strip	Mean diameter measure in mm (Adjusted: 2a – 5 = D.M.)	22.0	23.2	20.6	19.8	18.2	22.8
Disk	Mean diameter measure in mm	30.5	33.0	21.7	28.2	23.0	24.0
	Disk unit conversion term	8	10	1	8	5	0

the concentrations for streptomycin were the same in both methods no conversion term should be added.

For practical purposes the disk units obtained may be used for comparison of the two methods. It is understood by the author that theoretically there are other diffusion factors which might have been considered had it been feasible.

After classifying the disk unit figures into Ericsson's four sensitivity groups, the correlation coefficients for the six agents were computed and rated according to the following scale:

Bad correlation ..... = > + 0.4
Fairly good correlation = + 0.4 to + 0.7
Good correlation. .... = < + 0.7
Full correlation ..... = + 1.0

These disk unit figures comprise Fig

DISCUSSION

The errors of the method are reasona consistent for sulfathiazole, penicillin, ch tetracycline and oxytetracycline when strip and disk figures are compared. In case of the chloramphenicol, the lower c centration in the strips may explain discrepancy; perhaps a higher initial c centration of chloramphenicol could c pensate this difference observed.

On the whole the errors of the meth compare favorably with each other

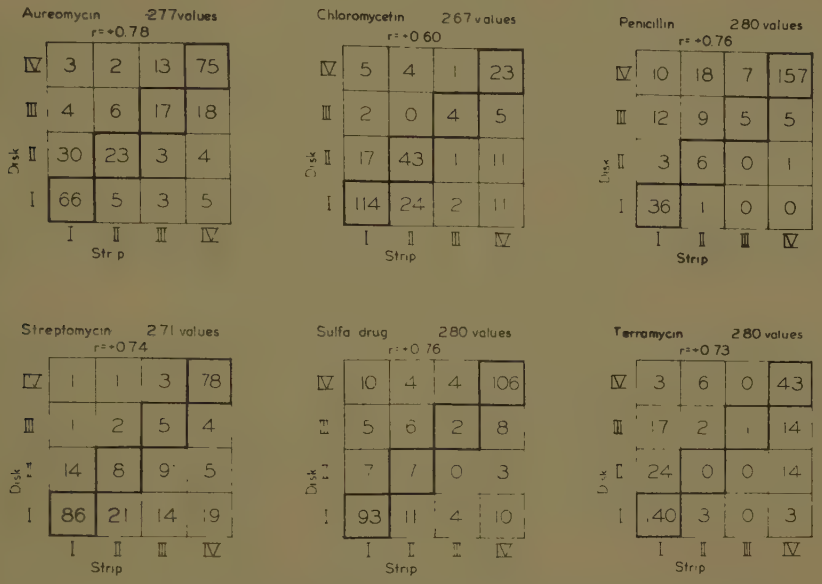

Fig. 3. Worksheet showing spread of values of 280 strains and correlation coefficients for the six antibiotic agents.

gh the actual strip readings of inhibition onsiderably lower than those of the disk od.

ie correlation coefficients are "Good" all antibiotics except chloramphenicol, "Fairly Good" for this agent. This er correlation, like the difference entered in the error of method for this iotic, may be ascribed to the aforeioned cause. The inherent variations of method make it difficult to obtain a er correlation and it is justified to say the methods have about the same reity.

COMMENTS

ie two methods are similar in accuracy sensitivity. Synergism and antagonism pparent using either method, but may served with more acuracy with the strip method. Also the strip method has some practical advantages. Only one 10-cm plate is required to test a strain against 6 antibiotics. Placing a single strip on a plate is considerably easier and quicker than handling 6 separate disks for each determination. Since the readings are always made perpendicular to the strip, no time is necessary to judge the axis of greatest inhibition.

As a routine technique, however, the primary advantage of the strip method over the disk method is the possibility of using a standard strain of known sensitivity for comparison purposes in each assay — whereby the test strain can be classified as "resistant", "fairly sensitive" or "sensitive". The standard strain also serves as a control of the intrinsic variations in strips, media and technique usually encountered. The investigation did not utilize the standard strain as a reference strain, but

the author believes that the clinical need of such a method of sensitivity determination justifies its special mention. However, it is obvious that neither method can compensate for variations in inoculum size and intensity of growth.

SUMMARY

Two diffusion methods of estimating bacterial sensitivity to antibiotics have been compared. It is shown that the two methods are about equal with regard to sensitivity and accuracy. Some practical advantages for laboratory use of the strip method are mentioned.

REFERENCES

Czerkinsky, G., Diding, N. & Ouchterlony, Ö: A new method for determination of bacteri sensitivity to chemotherapeutic agents by meas of paper strips. Scandinav. J. Clin. Lab. I vestigation *7*, 1955.

Ericsson, H., Högman, C. & Wickman, K.: A pap disk method for determination of bacteri sensitivity to chemotherapeutic and antibiot agents. Scandinav. J. Clin. Lab. Investigatio *6*, suppl. 11, 1954.

Erlansson, P.: Determination of the sensitivity vitro of bacteria to chemotherapeutic agent Acta path. et microbiol. Scandinav., suppl. 8 1951.

Wilks, S. S.: Elementary statistical analysis. Pri ceton University Press, Princeton, N. J., 194

Croxton.: Elementary statistics with applicatio in medicine. Prentice Hall Inc., New Yor 1953.

nav. J. Clin. & Lab. Investigation 21—28, 9, 1957.

THE DEVELOPMENT OF THE SERUM ELECTROLYTE NCENTRATION IN NORMAL INFANTS AND CHILDREN*

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(Received for publication July 25, 1956)

e chemical pattern of the blood of nor-nfants and children has been the subject veral investigations. However, in only rather limited parts of this field has umber of subjects examined been suf-t for results of statistical significance obtained. Still less information is avail-oncerning the correlation between the the child and the chemical composition blood.

serum electrolytes comprise one of elds which have received little attention child. Yet, due to their clinical im-nce, it would be of value to know the ntration and standard deviation of n, potassium and chloride in the serum healthy child. Further, a study of how ormal mean values change as the child ps would be of ontogenetic and physio-l interest.

reason why these figures have not sufficiently studied is probably that methods of analysis were laborious equired comparatively large amounts of . These difficulties have now been me by the introduction of micro-titri- and flame-photometric methods.

he investigation was aided by grants from earch allowances of the City of Stockholm.

This paper presents flame-photometric analyses of sodium, potassium and calcium and micro-titration of chloride in serum obtained from 276 apparently healthy children and infants, newborn and up to the age of six years. The results have been statistically treated.

It would have been of interest to widen the investigation to include several other serum concentrations such as bicarbonate, magnesium, *etc.* However, as it was impossible to take larger quantity of blood from the small children, we had to confine ourselves to a few determinations, and to the study of protein fractions and serum cholesterol described in the following paper.

MATERIAL

All the newborn babies were from the maternity wards of St. Erik's Hospital. They were all full-term. The other children were from different infants' and children's welfare centres belonging to the community of Stockholm. All the subjects were examined by pediatricians, and samples were taken only from infants and children who were considered to be in good health. We are aware of the possibility that a disease may have escaped detection in a few cases.

In order to obtain results which would be suitable for statistical analysis, we decided to fix in advance the age-groups from which samples would be taken. Nevertheless, when the investigation was

Table I. *Highest and lowest aberrations from the intended age of the groups in th material.*

Intend. age ..	1 day	3 m	6 m	9 m	12 m	18 m	3 y	6 y
Days above ..	6	15	18	12	21	30	48	180
Days below ..	0	18	16	7	15	8	91	30

completed it turned out that in a few of the groups some of the results were insufficient for statistical treatment, and in some other small groups the standard deviations became comparatively high.

In fixing the age groups, the following factors were taken into account: the growth and development of normal infants and children; the incidence of the usual infections, and the degree of resistance to infection which is present at various ages; and the changes in the diet as the child grows older.

The age-groups chosen were: the first week of life, three, six, nine, twelve and eighteen months, and three and six years. Table I shows the maximum aberrations from the chosen ages which ocurred in the material. It should be pointed out that very few of the subjects reached these extremes; nearly all were within a few days (in the case of those aged up to 12 months) or a few weeks (in the case of the older children) of the intended age. The sex of the children was not taken into account; boys and girls were present in about equal numbers.

For comparison, and as a control on the methods, serum from a number of healthy adults (students and laboratory staff) was examined in the same manner and by the same technicians.

THE TAKING OF BLOOD SAMPLES

In infants of an age up to twelve months the blood was taken by pricking one of the great toes with a sharp lancet. The sample was used only if the blood ran amply from the wound. About 2 ml was collected in a small Ellerman tube. From the older children the blood was taken by pricking a finger tip, and collected in the same manner. The blood was immediately brought to the laboratory and centrifuged, and the serum was decanted without delay.

All samples showing even a trace of visible hemolysis, clotting or opacity were discarded. Because of that we had to reject a great number of samples, especially from the newborn infants. The analy were carried out on the same or the following d

We realise that venous or arterial blood wo have been preferable. However, out of considerat for the children, their parents, and the person we restricted ourselves to arteriolar blood obtai by pricking.

ANALYTICAL METHODS

Sodium, potassium, calcium: Weichselbau Varney flame-photometer according to the ma facturer's instructions.

Chloride: Brun's modification of the met of Schales & Schales (1949).

All analyses were carried out in duplicate.

STATISTICAL METHODS

The statistical calculations were carried out cording to current methods.

We have calculated with a confidence of 95 cent and with a p-value of 90 per cent. (90 per c of normal values will fall within the calcula limits, and the chances are 95 per cent that th limits are valid).

We have had the following conditions for sig ficance of differences:

		label
$p > 0.05$	no significance	—
$0.05 > p > 0.01$	probably significant	*
$0.01 > p > 0.001$	significant	**
$0.001 > p$	highly significant	***

where p is the probability that the difference random result.

If the figures for the mean values plotted aga age did not appear to form a curved line, the e tion of the regression line in the form:

$$y = b\,x + k$$

was calculated according to the method of squares.

ble II. *The results of the analysis of Na, K, Ca and Cl in serum from healthy nts. Concentrations are given in milliequivalents per liter serum; n = number of cts examined, x = mean value, s = standard deviation, εx = standard error of mean, m = months, y = years.*

	Newborn	3 m	6 m	9 m	12 m	18 m	3 y	6 y	Adults	
									men	women
n	95	22	53	35	27	15	11	13	31	30
x	139.29	135.13	133.74	133.48	135.00	136.00	132.65	129.48	131.63	130.03
s	8.66	7.83	6.50	7.61	10.65	5.56	7.00	5.26	5.46	5.10
εx	0.89	1.56	0.89	1.29	2.05	1.43	2.09	1.43	0.98	0.94
n	95	12	43	32	24	13	10	12	31	30
x	4.65	5.24	5.04	5.03	4.83	4.72	4.16	4.23	4.28	4.54
s	0.43	0.47	0.59	0.51	0.48	0.59	0.84	0.51	0.35	0.49
εx	0.04	0.14	0.09	0.09	0.09	0.16	0.26	0.15	0.06	0.09
n	-	11	26	21	10	6	3	4	31	30
x	-	12.04	11.30	11.54	11.66				10.30	10.01
s	-	1.31	1.23	0.77	1.06	-	-	-	0.72	0.50
εx	-	0.39	0.24	0.17	0.33	-	-	-	0.13	0.09
n	95	20	57	34	26	16	11	13	30	
x	107.70	113.55	108.76	108.59	107.77	103.54	101.14	103.37	102.97	
s	8.14	15.23	14.18	12.95	13.50	16.51	9.34	9.69	2.54	
εx	0.84	3.41	1.88	2.22	2.65	4.63	2.83	2.65	0.46	

significance of the slope of the regression s calculated by analysis of variance.

statistical treatment of the results has been out by Dr. Staffan Ekblom of the Statistical h Group of the University of Stockholm.

RESULTS

mean values of the results of analyses own in Table II. In only four of the of infants was the number of calcium sufficient for statistical treatment.

e III shows the limits within which cent of the normal values will fall confidence of 95 per cent. As our l groups of three- and six-year-old children were precariously small we have put these two together in this table. This seemed justifiable as the results from the two groups were very similar.

As we found obvious differences between age groups for some mean values, the regression was examined. It was considered probable that the changes in the chemical pattern would be most pronounced shortly after birth. Therefore we have compared the newborn babies with the infants of three months, and the latter with the children six years of age. Finally, the six-year-old children have been compared with the adults. The results are shown in Table IV.

Table III. *The limits within which 90 per cent of the results from normal subjects will be found with a confidence of 95 per cent, provided the same methods are used, and with reservation for deviations due to race, climate, etc.*

		Newborn	3 m	6 m	9 m	12 m	3+6 y	Adults
Na	Upper limit.....	154.6	150.7	145.7	148.4	157.2	144.2	141.1
	Lower limit.....	122.0	117.5	119.9	116.6	110.8	115.7	118.5
K	Upper limit.....	5.46	6.48	6.25	6.11	5.90	5.69	5.32
	Lower limit.....	3.84	3.99	3.84	3.95	3.75	2.69	3.01
Ca	Upper limit.....	-	-	13.99	13.30	-	-	11.45
	Lower limit.....	-	-	8.61	9.78	-	-	8.86
Cl	Upper limit.....	109.2	148.7	136.7	135.8	137.4	121.0	108.4
	Lower limit.....	106.2	78.4	80.9	81.4	78.2	80.9	103.0

Table IV. *The differences and their significance between the mean values for the newborn infants and children 3 months old and 3—6 years old, and between the latter group and adults. The differences are given in milliequivalents per liter serum.*

	0—3 m	Signific.	3 m—(3+6) y	Signific.	(3+6) y—ad.	Signific.
Na	4.16	*	4.21	*	0.09	-
K	—0.59	* * *	1.05	* * *	—0.22	-
Cl	—5.94	*	12.63	*	—2.05	-

We found a significant increase in the serum potassium between birth and the age of three months, and a subsequent slow decrease back to a mean value which was below that of the newborn infants and similar to the adult value. The first increase and the subsequent decrease are highly significant.

The chloride values followed a course very similar to the potassium values. At the age of 18 months the mean value was within the normal range for adults. The deviations were very high for the chloride figures, and the differences are only probably significant but the slope of the regression line is highl significant.

The sodium values followed anoth course. With the exception of a slight te dency to recovery at the ages of 12 and months, they fell from birth to six years age. This decrease was steepest during t first three months but it was never mo than "probably significant".

The calcium values were too few for c culation of regression. Mean values we calculated for the ages of three, six, nine a twelve months only. The mean at thr

onths was higher than that in the older ildren. Otherwise the respective means peared to be rather similar and they did t seem to be very different from the mean r adults.

DISCUSSION

In some of the groups (K — three years, a — twelve months, Cl — three months) e standard deviation was remarkably high d in all the groups it was higher than that the adults. This is not surprising as each e-group may consist of children at quite fferent stages of development. Thus the aterial in each group must have been much ss homogeneous than a group of adults. This xplanation is supported by the fact that the andard deviations were comparatively low the newborn babies, who can be considered a relatively homogeneous group. At the e of three months the deviations were very gh, but at six years they again approached e deviation in the adults.

We cannot completely exclude the possiility that in a few cases a very slight hemoysis may have escaped our observation. If is has occurred it may have increased the ean potassium concentration slightly, due the high potassium concentration in the rythrocytes, but it may also have increased e standard deviation considerably. On the her hand it is not likely that a slight degree f hemolysis would be of much influence on e chloride figures, and its influence on the odium values must be very small.

There is a pitfall in our comparison beween the results from the children and those om adults. The adults' blood was taken by enipuncture while the children's blood was from prick-wounds. This may have incurred some difference.

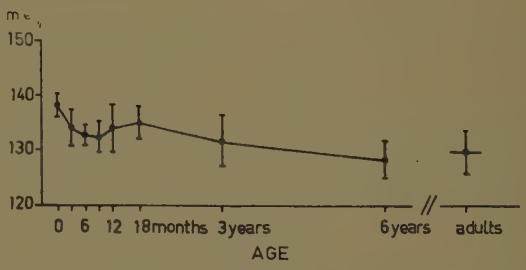

Fig. 1. The mean values for serum sodium concentration in milliequivalents per liter. The vertical lines represent the limits for a confidence of 95 per cent.

In spite of the comparatively high standard deviations and of the fact that the age of a child does not necessarily correspond to its state of development, the results make it possible to follow the course of the electrolyte pattern of the serum during the progress of growth. The figures in Table II show that at the age of three years the concentration of sodium, potassium and chloride had reached normal values for adults.

At birth the concentration of sodium, potassium and chloride was above that of the adults. Potassium and chloride rose still more during the first three months of life. After that they decreased slowly until at the age of three years they had reached the adult level. The sodium concentration, on the other hand, did not show any initial increase. At the age of three years it had decreased to the adult value. The courses are demonstrated in Fig.s 1, 2 and 3. These figures also include the regressions lines between three and eighteen months, calculated by the method of least squares. The few means for calcium are not illustrated.

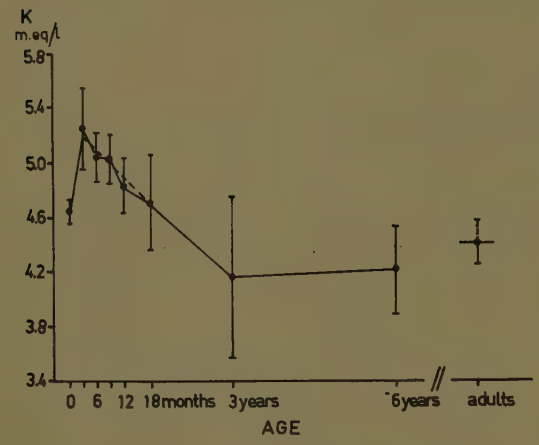

Fig. 2. The mean values for serum potassium concentration. Legend as for Fig. 1.

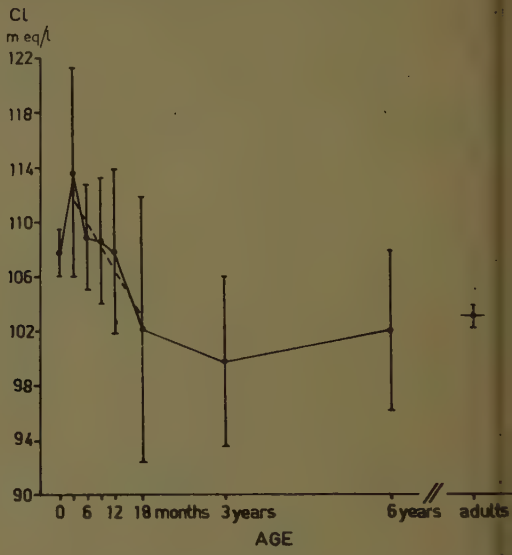

Fig. 3. The mean values for serum chloride concentration. Legend as for Fig. 1.

Table IV shows the statistical significance of some of the found differences. We have chosen to examine three differences for each electrolyte (except calcium), namely 1) between the newborn and the infants three months old, 2) between the latter and the children of three and six years of age counted as one group, and finally 3) between this pooled group and the adults. We selected the first difference because the first three months of life involve the most dramatic part of the development of the infant. The second difference was chosen as representing the difference between serum composition in infancy and in childhood — the latter being similar to that of the adults. As this level was reached at the age of three years and as it was practically the same at six years, these two groups were treated as one in the calculation of differences.

Gottfried, Bogin & Levycky (1954) analyzed serum from 35 normal full-term newborn infants and found sodium and chloride concentrations still higher than ours. The did not determine potassium and calcium and they made no comparison betwee children of different ages. Rada (1955 determined sodium and potassium in serum from normal children aged up to one year He compared infants one to six months ol with those aged 6 to 12 months. For bot ions the concentration was higher in the firs group than in the second. For sodium th difference was negligible; for potassium was considerable — from 5.19 to 4.80. Th results presented here confirm those of th authors mentioned.

At the present we cannot explain why th electrolyte concentrations are high at birth They are not increased in the parturien woman. The high and increasing potassium figures might be explained by the fact tha

uman milk is rich in potassium and poor sodium; on the other hand the milk is oor in chloride too, and yet chloride shows e same course as potassium. All other atmpts at an explanation would be mere uesswork. It may be observed that the most ovious changes in this material, those for and Cl, occur at the age of three months nd three years — not at the same time when ost infants are weaned, nor when they start walk.

Corsa, Gribetz, Cook & Talbot (1956) etermined the total sodium and potassium infants by means of radioisotopes. They ound that up to an age of eighteen months e total sodium of the infants was below at of the adult while total potassium had e adult value already at birth (when calulated per kg body weight).

According to Josephson & Dahlberg 1952), serum sodium and chloride concenation continues to decrease throughout life hile potassium is nearly stable. The investiation of Josephson & Dahlberg was carried ut with methods now abandoned and therore their mean values are not to be comared with the ones presented here. Howver, it seems as if the decrease has already egun at the age of three months.

Calcium was not determined in the blood om the newborn infants. At the age of ree months it was definitely above the noral level, but at six months the average was ready 11.30. It stayed near this figure up twelve months. It was not examined in der children. It is generally considered that rum calcium is a little higher in infancy an in the adult and that it reaches the adult lue during adolescence. This is confirmed by our small material. Even the mean values from children three and six years of age were above what we found in adults.

The limits given in Table III may be of some practical value to the clinician as 90 per cent of normal values will fall within them.

SUMMARY

The serum concentration of sodium, potassium, calcium and chloride was determined in normal infants and children of the following age groups: newborn, three, six, nine, twelve, and eighteen months, and three and six years.

In the newborn infants the concentration of all three electrolytes was above that of normal adults.

Potassium and chloride concentration increased steeply to the age of three months, but after that they decreased slowly until at the age of three years they reached the normal values of adults.

Sodium concentration decreased (with some insignificant interruptions) to reach the adult value at the age of six months.

Calcium was determined only in the groups aged three, six, nine and twelve months. The mean was highest in the three months group but the values were too few for comparison of the means.

REFERENCES

Brun, C.: Klorbestemmelse; en mikrometode. Nord. Med. *42*, 1774, 1949.

Corsa, L., Gribetz, D., Cock, C. & Talbot, N.: Total body exchangeable water, sodium and potassium in "hospital normal" infants and children. Pediatrics *17*, 184, 1956.

Gottfried, S. P., Bogin, M. & Levycky, N. V.: Blood and electrolyte studies in normal newborn full-term babies. Am. J. Dis. Child. *87*, 543, 1954.

Josephson, B. & Dahlberg, G.: Variations in the cell-content and chemical composition of the human blood due to age, sex and season. Scandinav. J. Clin. Lab. Investigation *4*, 216, 1952.

Josephson, B. & Gyllenswärd, C.: The develo ment of the protein fractions and of cholester concentration in the serum of normal infan Scandinav. J. Clin. Lab. Investigation *9*, 2 1957.

Rada, O. B.: Normal values of serum potassiu and sodium in children and their variation dystrophy. Anales fac. med. Univ. nace. may San Mario, Lima, *37*, 755, 1955.

andinav. J. Clin. & Lab. Investigation 29—38, 9, 1957.

HE DEVELOPMENT OF THE PROTEIN FRACTIONS AND OF CHOLESTEROL CONCENTRATION IN THE SERUM OF NORMAL INFANTS AND CHILDREN*

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In the preceding paper (Gyllenswärd & osephson, 1956) we have pointed out the arcity of data on the chemical composition the blood of normal children.

The pattern of serum protein fractions has en more carefully examined than other rum constituents of the infant, but there is ill a lack of systematic investigation of how is pattern changes with the age of the ild. This paper presents an attempt to fill this gap. At the same time the serum olesterol has been determined as it is an- her organic serum constituent of interest connection with the proteins. Thymol tur- dity tests were also performed. The mate- al was normal infants from one day old d up to an age of six years.

These determinations were chosen not ly because they were of clinical and physio- gical interest but also because they could carried out on very small quantities of rum. It would have been worth while to udy the concentration of several further nstituents such as lipids, urea, uric acid, eatinine, phosphatases, *etc.* However, it rned out to be difficult to obtain enough serum even for the determinations described here, and the other examinations have had to be postponed to a later study on new material.

MATERIAL AND SAMPLE-TAKING

The same blood samples were used as in the preceding paper (see Table I, Gyllenswärd & Josephson, 1957).

ANALYTICAL METHODS

Total serum proteins were determined by the biuret method, modified according to Josephson & Andurén (1949). The protein fractions were determined by paper electrophoresis, using the apparatus of Dettker & Andurén (1950), and the proteins were stained with Ponceau Red according to Röttger (1951). We did not use either of the common methods of obtaining the electrophoresis results, *i. e.* cutting the paper strips into a great number of equal parts or reading the migration curve directly from the paper strip. We preferred to draw a fine pencil-line between the protein fractions and to cut them apart with a fine pair of scissors. With this method there is no difficulty in isolating the fractions as they are well separated from each other and have quite sharp boundaries. Each fraction was extracted with 0.02 N Na OH solution from its respective strip-part and read in a Beckman B photometer at 500 mμ.

The thymol turbidity test was carried out on 0.05 ml serum according to Shank & Hoagland's (1946) modification of MacLagan's method.

* The investigation was aided by grants from e research allowances of the City of Stockholm.

Table I. *The results of the analysis of total protein, protein fractions (separated by paper electrophoresis) and cholesterol in serum from healthy infants. Concentrations of total proteins are given in g and of cholesterol in mg per 100 ml. The protein fractions are reported as a percentage of the total protein. n = number of subjects examined, x = mean value, s = standard deviation, ε = standard error of mean, m = months, y = years.*

		New-born	3 m	6 m	9 m	12 m	18 m	3 y	6 y	Adult
Total protein (TP) gr per 100 ml	n	92	21	51	33	28	16	11	13	28
	x	7.16	6.86	7.08	7.08	7.22	7.16	7.20	7.17	7.6
	s	0.96	0.74	0.74	0.66	0.75	0.54	0.70	0.46	0.4
	εx	0.10	0.16	0.10	0.12	0.14	0.13	0.21	0.13	0.0
Albumin in per cent of TP	n	58	23	53	34	27	16	11	13	30
	x	65.56	61.49	62.72	60.70	62.14	62.44	62.36	61.88	63.2
	s	3.18	5.21	5.85	6.12	6.00	5.57	3.70	5.10	2.6
	εx	0.42	1.09	0.80	1.05	1.15	1.39	1.12	1.41	0.4
α_1-globulin in per cent of TP	n	58	23	53	34	27	16	11	13	30
	x	5.17	6.08	5.52	5.71	5.31	5.20	5.17	5.01	5.5
	s	1.88	1.74	1.79	1.66	1.54	1.53	1.45	1.00	0.8
	εx	0.25	0.36	0.25	0.28	0.30	0.38	0.44	0.28	0.1
α_2-globulin in per cent of TP	n	58	23	53	34	27	16	11	13	30
	x	7.13	11.51	11.90	11.94	11.48	11.54	10.75	8.91	6.7
	s	1.61	2.65	2.52	2.46	2.38	2.90	2.71	1.61	0.6
	εx	0.21	0.55	0.35	0.42	0.46	0.73	0.82	0.45	0.1
β-globulin in per cent of TP	n	58	23	53	34	27	16	11	13	30
	x	7.45	10.33	9.82	10.86	10.12	10.09	9.46	9.94	9.3
	s	2.12	1.95	1.89	1.56	2.05	1.06	1.31	1.38	1.1
	εx	0.28	0.41	0.26	0.27	0.40	0.26	0.39	0.38	0.2
γ-globulin in per cent of TP	n	58	23	53	34	27	16	11	13	30
	x	14.63	10.59	10.15	10.80	10.89	10.72	12.25	14.27	15.0
	s	2.23	3.37	3.05	3.51	3.09	2.86	1.42	2.54	2.45
	εx	0.29	0.70	0.42	0.60	0.59	0.71	0.43	0.71	0.1
Albumin/globulin ratio	n	57	23	53	34	27	16	11	13	30
	εx	1.93	1.65	1.74	1.59	1.68	1.69	1.65	1.65	1.7
	s	0.30	0.39	0.44	0.44	0.39	0.39	0.22	0.42	0.2
	εx	0.04	0.08	0.06	0.08	0.08	0.10	0.07	0.12	0.0
Cholesterol mg/100 ml	n	79	17	46	31	26	10	8	11	30
	x	191.3	187.2	185.0	189.5	200.1	212.2	-	221.8	238.5
	s	51.0	44.8	54.5	41.3	49.5	47.8	-	41.4	43.5
	εx	5.7	10.9	8.0	7.4	9.7	15.0	-	12.5	7.9

Cholesterol was determined on 0.2 ml serum according to Pearson, Stern & McGavack (1952), with the modification that the color was read at its maximum, 625 mμ instead of 500 mμ. In our hands this method gives results coincident with t Bloor methods.

All tests except the electrophoresis were carri out in duplicate.

able II. *Shank-Hoagland Units of thymol turbidity in serum from healthy infants. n = number of subjects examined.*

Units	Newborn	3 m	6 m	9 m	12 m	18 m	6 y
n	93	24	53	34	26	14	12
0	68	1	5	3	1	4	1
1	14	9	14	10	6	7	7
2	8	8	17	13	12	2	1
3	3	5	9	6	4	1	1
4	-	1	7	2	2	-	-
6	-	-	-	-	1	-	-
14	-	-	1	-	-	-	-

The statistical methods were the same as in the ceding paper. The statistical calculations were ried out by Dr. Staffan Ekblom of the Statis-l Research Unit of the University of Stockholm.

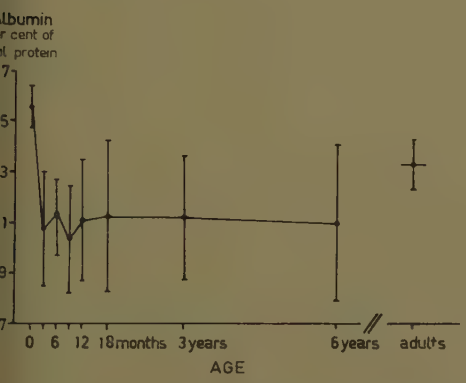
RESULTS

The number of subjects examined, the an values, standard deviations and stand-l errors are given in Tables I and II.

Total serum proteins: the means for all groups were very similar. The means for the age groups were lower than the lult" value.

Albumin (Fig. 1): the newborn infants had a mean value slightly above that for adults. After three months the albumin figures were already within the "adult" range and there they stayed.

The α_1-globulin (Fig. 2) started lower than "adult", increased during the first three months of life and then returned slowly to adult concentration and lower in about one



. 1. The mean values of the percentage of serum mmin for different ages. The vertical lines resent the limits for a confidence of 95 per cent.

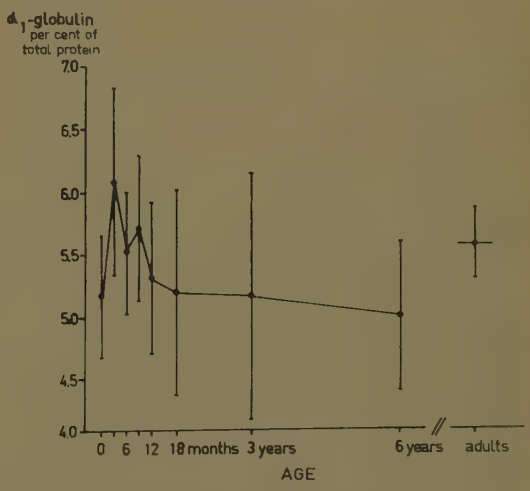


Fig. 2. The mean values of the percentage of α_1-globulin. Legend as in Fig. 1.

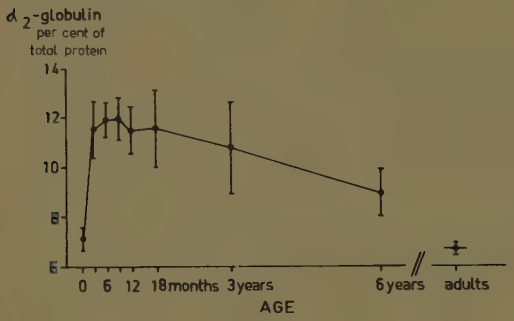


Fig. 3. The mean values of the percentage of α_2-globulin. Legend as in Fig. 1.

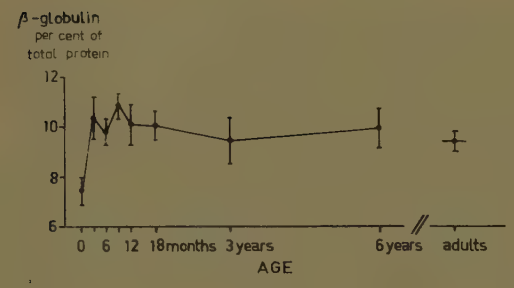


Fig. 4. The mean values of the percentage of β-globulin. Legend as in Fig. 1.

to three years. The differences were only "probably significant".

The α_2-globulin (Fig. 3) was normal at birth but increased considerably during three to six months. At the age of eighteen months α_2 started to decrease slowly, but at the age of six years it was still higher than in the adults. The differences between the mean concentration at birth and at the age of three months, and between three months and six years, were significant.

The β-globulin (Fig. 4) started low, then increased to a comparatively high value, after which it went down slowly to reach the "adult" value at three years of age.

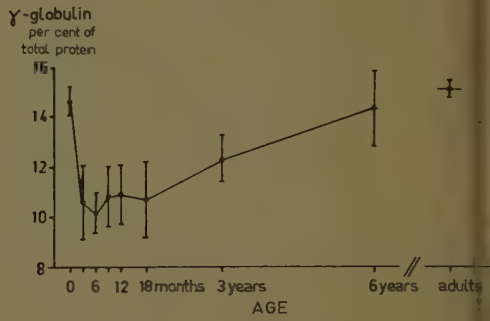
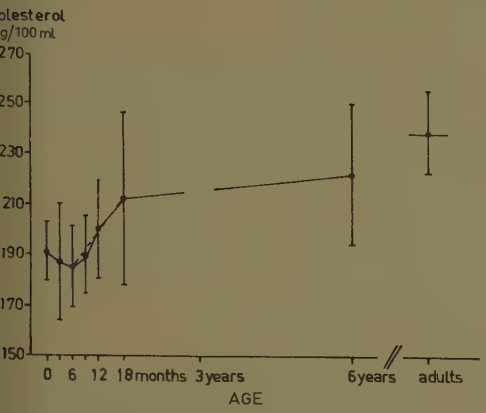


Fig. 5. The mean values of the percentage of γ-globulin. Legend as in Fig. 1.

The γ-globulin (Fig. 5) followed a cour quite opposite to that of the other globulir At birth the mean was similar to the me for adults, but already at the age of thr months it had decreased significantly. stayed on the new lower value until the a of eighteen months and then rose. At the a of six years it was still a little below t normal value for adults.

The $\frac{\text{albumin}}{\text{globulin}}$ ratio was higher at bir than in adults but already at the age of thr months it had reached the adult value a there it stayed practically constant.

Thymol turbidity: As in the material Josephson & Dahlberg (1952) the distrib tion was rather skew. For that reason have not calculated the mean values but ha given all the results in Table II. It appe that at birth most children had a very l value, but at the age of three months distribution was already similar to that of t adults. After that there was no signific change. In the group of three year-o children this reaction could be carried only on eight subjects. Hence this group not included in the table. Its distribution w



g. 6. The mean values of the serum cholesterol ncentration for different ages. The dotted line the regression line between the ages of 6 and 18 months. Legend otherwise as in Fig. 1.

ormal and similar to what Josephson & ahlberg found in adults.

The serum cholesterol (Fig. 6) in all the oups had a mean below that of the adults. was low at birth and stayed low for nine onths, but at the age of twelve months it arted rising slowly and it continued to rise the way up to the six-year group. The ree-year group was too small to be con-dered. However, the deviations were high d the differences are not significant.

The differences and their significance are ven in Table III.

DISCUSSION

In contrast to most other serum consti-ents in infants and children, the total pro-in concentration and the concentration of e different protein fractions have been the bject of several investigations. As far as e have found, however, no systematic rvey of children of different ages has been blished — at least not on a group big enough for statistical significance. The main results of some of the most careful investigations are summarized in Table IV.

In Table V are given the limits within which 90 per cent of the values will turn out with a confidence of 95 per cent. Due to the high standard deviations most of the limits are surprisingly wide. One reason for this may be the limited number of subjects examined. Another reason may be the highly varying degree of development in infants of the same age.

Previous investigators generally found that newborn children had a low serum-protein concentration which increased rapidly with the age of the child. Our results are not in agreement with this. On the contrary, we found the value nearly constant in all the ages examined although the mean found in the children was definitely below that of the adult. It may be pointed out that all the children in our material were well nourished.

The results found by previous investigators on the partition of the protein fractions are conflicting (see Table IV); our values differed significantly with the age of the subjects. The albumin portion decreased during the first three months of life (from about 65 to about 62 per cent) but then it stayed constant. Calzolari & Orlandi (1955) found in a small material that serum albumin decreased appreciably during the first days of life. It seems possible that the newborn child has some circulating albumin transferred from the placenta. After three months this surplus has disappeared.

The α_1-globulin did not show much variation and had a value similar to the adult one in all the age groups. This was in contrast to the α_2-globulin which at birth had a con-

Table III. *The difference and their significance between the mean values for the newborn infants and children 3 months old, between children 3 months old and (3 + 6) years old and between the latter ones and adults. The differences are given in the same units in Table I.* *** = *highly significant,* ** = *significant,* x = *probably significant,* 0=*no significance.*

	0—3 m		3 m—(3+6) y		(3+6) y—adults	
	Diff.	Signific.	Diff.	Signific.	Diff.	Signific.
Total protein ..	0.300	0	—0.326	0	—0.510	**
Albumin	4.07	***	—0.613	0	—1.18	0
α_1-globulin	0.91	*	1.00	*	—0.49	0
α^2-globulin	—4.38	***	1.76	*	3.05	***
β-globulin	—2.88	***	0.61	0	0.34	0
γ-globulin	4.04	***	—2.75	**	—1.73	**
A/G ratio	0.279	***	—0.002	0	0.073	0
Cholesterol	4.1	0	—34.8	*	—16.5	0

centration slightly above that of the adults but increased markedly during the first three months to a value nearly twice that of the adults. It stayed on this level until the age of three years, when it started going down. At the age of six years it had not yet reached the adult value. As acute infection is the most common cause of an elevated α_2-concentration in adults it is tempting to attribute the high values in the infants of three months and older to the relatively high frequency of acute infections in infants' and children's homes and centres. Such an elevation of α_2 may have persisted in spite of the fact that the subjects appeared healthy when the samples were taken.

The β-globulin was low in the newborn infants, but in three months it increased to a value above that of the adults. At the age of eighteen months it still had the same concentration, while at three years it had fallen to the adult level. The first increase was highly significant, the subsequent slow decrease was not even "probably significant" As the β-globulins consist to a large exte of lipo- and gluco-proteins, the low level i the newborn infants may be explained by deficient synthesis during fetal life. Afte three months the synthetic capacity (of th liver?) would be normal.

The curve of development of the γ-globu lins formed a mirror-image of that of the α At birth they had a concentration similar t that of the adults but already at three month they had decreased considerably and sign ficantly. An increase was not observed unt the three-year group, and at six years th adult concentration was reached. The reaso for this behavior is obscure. As pointed o by Körver (1953), the low values observe in most of the infant groups are not far fro what is found in clinical cases of hypogam maglobulinemia, and this may be one e planation of the susceptibility of infants infections. Not until the age of six yea does the child seem to have "learned how

Table IV. *Some published investigations on the concentration of protein fractions in serum from normal infants. All are from small groups of material. In the first two references the concentration figures denote g per 100 ml, in the others per cent of total serum proteins. w = weeks, m = months, y = years.*

Author	Year	Albumin	α_1-globulin	α_2-globulin	β-globulin	γ-globulin
Darrow & Cary	1933	birth 3.7 infants 4.3 slowly increasing during childhood	birth 1.8, infants 2.0 per cent increasing during childhood			
Trevorrow, Kaser, Patterson & Hill	1941/42	birth 3.8 3 months 4.7 6—12 months as adults	birth 1.7, 2 months 1.3, 6 months 1.3, 4 years 2.0			
Lubschez	1948	2—4 y 62 5—7 y 57	2—4 y 4.5 5—7 y 5.2	2—4 y 9.1 5—7 y 9.7	2—4 y 14.3 5—7 y 12.2	2—4 y 7.2 5—7 y 11.0
Knapp & Routh	1949	newb. 59 6—4 w 62 2—4 y 61 5—11 y 57	newb. 7.4 6—4 w 6.7 2—4 y 6.3 5—11 y 6.2	newb. 7.7 6—4 w 12.9 2—4 y 10.7 5—11 y 9.8	newb. 9.5 6—4 w 11.5 2—4 y 11.3 5—11 y 12.0	newb. 13.0 6—4 w 3.3 2—4 y 7.2 5—11 y 9.7
Homolka & Mydlil	1955	newb. 54 3 m 51 6 m 57 12 m 49	newb. ..... 13.5 3 months ..... 14.5 6 months ..... 14.1 12 months 21.0		newb. 22.5 3 m 28.0 6 m 24.9 12 m 25.4	newb. 11.0 3 m 6.1 6 m 4.4 12 m 4.4
Calzolari & Orlandi	1955	newb. 62 8 days 51	irregular	irregular	newb. 7 8 days 15	newb. 23 8 days 18
Sternberg, Dagenais-Perusse Dreyfuss	1956	newb. 51	newb. 3.6	newb. 11.6	newb. 14.5	newb. 19.0 2 m 3.5

nthesise antibodies". The high value at rth may be due to transfer of γ-globulin om the placenta.

This reciprocal behavior af α_2 and β on e side and γ-globulins on the other has en observed earlier on small groups of terial by several investigators (Martin du n & Moore, 1948; Knapp & Routh, 1949; Grell, 1950; Homolka & Mydlil, 1955; Sternberg, Dagenais-Perusse & Dreyfuss, 1956). Calzolari & Orlandi (1955) found this reciprocal behavior to occur already during the first week of life.

The fact that the ratio $\frac{\text{albumin}}{\text{globulin}}$ was high at birth and reached the adult value at the

Table V. *The limits within which 90 per cent of the results from normal subjects will be found with a confidence of 95 per cent provided the same methods are used and with reservation for deviation due to race, climate etc. Units as in Table I.*

		New-born	3 m	6 m	9 m	12 m	18 m	3—6 y	Adults
Total protein	Upper l.	8.98	8.55	8.55	8.48	8.85	8.47	8.45	8.75
	Lower l.	5.34	5.16	5.61	5.68	5.59	5.85	5.91	6.63
Albumin	Upper l.	71.80	73.17	74.33	73.55	75.20	76.01	71.94	68.96
	Lower l.	59.32	49.81	51.11	47.85	49.08	48.87	52.26	63.28
α_1-globulin.....	Upper l.	8.86	9.99	9.08	9.20	8.65	8.93	7.53	7.29
	Lower l.	1.48	2.18	1.96	2.22	1.96	1.47	2.63	3.87
α_2-globulin.....	Upper l.	10.29	17.46	16.90	17.10	16.66	18.61	14.93	8.00
	Lower l.	3.97	5.56	6.90	6.78	6.30	3.34	4.57	5.40
β-globulin	Upper l.	11.62	14.71	13.57	14.14	14.59	12.69	12.69	11.79
	Lower l.	3.29	5.95	6.06	7.58	5.65	7.52	6.75	6.97
γ-globulin	Upper l.	19.01	18.15	16.20	18.17	17.62	17.68	18.47	20.31
	Lower l.	10.25	3.03	4.10	3.43	4.16	3.76	8.21	9.83
A/G ratio.....	Upper l.	2.51	2.53	2.62	2.51	2.54	2.63	2.39	2.17
	Lower l.	1.35	0.78	0.86	0.67	0.82	0.74	0.97	1.29
Thymol-turbidity ..	Upper l.	2	3	4	4	4	3	4	6
	Lower l.	0	0	0	0	0	0	0	0
Cholesterol	Upper l.	288.7	294.7	294.8	277.3	-	-	329.2	331.6
	Lower l.	94.0	79.7	75.2	101.7	-	-	114.8	145.4

age of three months is partly due to the changes in the albumin concentration. The same explanation may be valid for the preponderance of extremely low thymol-turbidity values in the newborn infants.

Several investigators have found a low cholesterol value in newborn infants. Sperry (1936) found the concentration to increase already during the first days after birth. In the large material of Hodges, Sperry & Andersen (1943) the mean concentration did not change much between the ages of two months and fourteen years. Rafstedt (1935) found a very low value in umbilical co blood, but also in infants a few days old was much below adult value.

We too found the serum cholesterol to b low in the newborn infants and it stayed lo during the first year of life. From that a it increased slowly. At the age of six years was still a little below the adult value. Th latter value is somewhat higher than the o usually given in the literature but similar the value, 239 mg per 100 ml, given b Josephson & Dahlberg (1952). This may b due to the fact that the method employed b

s is not quite specific (Zak & Ressler,1955). nfortunately we did not discover this un- ecificity until a considerable part of the ıvestigation was completed. However, the ifferences are small, and the mean values otained may be compared with one another. he low cholesterol values found in the small ıfants may be due to the comparatively low ıolesterol content of milk or to a defective ıolesterol synthesis.

In sum the results give the impression of defective protein and lipid formation in the ewborn infant. A great part of the γ-glo- ulin (and perhaps some albumin) may be "godmother's gift" from the placenta — a ift which is rapidly used up. Not until the ge of three to six years does the protein attern become identical with that of the dults. This hypothesis is supported by Berg- rand & Czar's (1956) discovery of a spe- ial protein fraction in serum from human etuses. However, this fraction was not ob- erved in the full-term babies of this ma- erial. A corresponding conclusion can be rawn for the development of the cholesterol. hus, it seems as if a defective synthesis uring infancy becomes "normalized" at the ge of three to six years.

SUMMARY

Total protein, protein fractions and chol- sterol were determined in serum from 265 ealthy infants, newborn and up to an age f six years.

Some statistically significant differences ere observed between the newborn infants nd those three months old. During these rst months of life albumin and γ-globulin decreased, while α_2- and β-globulin increased. During childhood α_2 was much higher than in adulthood while γ-globulin was much lower.

Cholesterol concentration was low at birth but increased slowly up to six years of age.

REFERENCES

Bergstrand, C. G. & Czar, B.: Demonstration of a new protein fraction in serum from the human fetus. Scandinav. J. Clin. Lab. Investigation *8*, 174, 1956.

Calzolari, G. & Orlandi, C.: Comportamento delle siero-proteine nel neonato sano. Riv. Italian. Ginecol. *38*, 134, 1955.

Darrow, D. & Cary, K.: The serum albumin and globulin of newborn premature and normal infants. J. Pediat. *3*, 573, 1933.

Dettker, A. & Andurén, H.: A time-saving easily-handled apparatus for paper electrophoresis. Scandinav. J. Clin. Lab. Investigation *6*, 74, 1950.

Grell: Ergebnisse elektrophoretischer Serumproteinbestimmungen bei Feten und Neugeborenen im Vergleich zum Erwachsenen. Zbl. f. Gynekologie *72*, 1827, 1950.

Gyllenswärd, C. & Josephson, B.: The development of the serum electrolyte concentration in normal infants and children. Scandinav. J. Clin. Lab. Investigation *9*, 21, 1957.

Hodges, R., Sperry, W. & Andersen, D.: Serum cholesterol values for infants and children. Am. J. Dis. Child. *65*, 858, 1943.

Homolka, J. & Mydlil, V.: Blood proteins in infants from a quantitative and qualitative point of view. Amer. J. Pediat. *129*, 129, 1955.

Josephson, B. & Andurén, H.: A method for the colorimetric determination of total serum proteins and of serum albumin and globulin. Acta Pediat. *38*, 335, 1949.

Knapp, E. & Routh, J.: Electrophoretic studies of plasma proteins in normal children. Pediatrics *4*, 508, 1949.

Körver, G.: Ergebnisse über Gammahypoglobulinämie. Klin. Wchnschr. *31*, 1036, 1953.

Lubschez, R.: Immunologic and biochemical studies in infants and children with special reference to rheumatic fever. Pediatrics *2,* 570, 1948.

Martin du Pan, R. & Moore, D.: Les protéines du sérum chez le foetus et le nourrisson de moins d'un an. Ann. Pediat. *171,* 290, 1948.

Metcoff, J. & Stare, F.: The physiologic and clinical significance of plasma proteins and protein metabolites. New England J. Med. *236,* 26, 1947.

Pearson, G. Stern, S. & McGavack, T.: Determination of total cholesterol in serum. Anal. Chem. *25,* 813, 1953.

Rafstedt, S.: Studies on serum lipids and lipoproteins in infancy and childhood. Acta Pediat. *44,* suppl. 102, 1955.

Röttger, H.: Ein neues Farbeverfahren der Serum-Eiweissfraktionen bei der Papierelectrophorese. Naturwissenschaften *39,* 451, 1951.

Schank, R. & Hoagland, C.: A modified metho for the quantitative determination of the thy mol turbidity reaction of serum. J. Biol. Chem *162,* 133, 1946.

Sperry, W.: Cholesterol content of the blood plasm in the neonatal period. Am. J. Dis. Child. *51,* 84 1936.

Sternberg, J., Dagenais-Perusse, P. & Dreyfuss, M. Serum proteins in parturient mother and new born. Canad. M. A. J. *74,* 49, 1956.

Trevorrow, V., Kaser, M., Patterson, J. & Hill, R. Plasma albumin, globulin and fibrinogen healthy individuals from birth to adulthood J. Lab. & Clin. Med. *27,* 471, 1941/42.

Zak, B. & Ressler, N.: Methodology in determination of cholesterol. Am. J. Clin. Path. *25,* 433 1955.

andinav. J. Clin. & Lab. Investigation 39—43, 9, 1957.

INORGANIC PHOSPHATE IN HUMAN ERYTHROCYTES[1]

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INTRODUCTION

The concentration of inorganic phosphate P_i) in human erythrocytes is usually con-lered to be lower than plasma P_i (Guest & apoport, 1941). The values obtained by dif-rent authors, however, vary from 0 to well ove plasma P_i. This will be apparent from able I which represents a compilation of ta from different workers. The tabulated lues for erythrocyte P_i have, where neces-ry, been recalculated to mg/100 ml P in erythrocytes. Most of the earlier work in this field was carried out on whole blood. Recalculation of these data is based upon an assumed hematocrit of 45 per cent and plasma P_i of 4 mg/100 ml. The listed values are consequently only approximations.

The early methods for determination of inorganic P in biological material were rather unreliable. Proper consideration to keeping samples cold before the analytical procedure was almost generally neglected and has prob-

Table I. *Inorganic P in erythrocytes; compilation and recalculation of data from the literature.*

f. No.	Method	Data in reference	Data recalculated to mg/100 ml P in red cells
4	Bloor (1918)	1.5 — 2.6 mg/100 ml H_3PO_4 in red cells	0.46— 0.82
11	Fiske & Subbarow (1925)	3.1 mg/100 ml P in whole blood	2.0
3	—»—	3.31— 5.03 mg/100 ml P in whole blood	2.5 — 6.3
17	—»—	2.4 mg/100 ml P in red cells	2.4
10	—»—	2.52—11.58 mg/100 ml P in whole blood	0.7 —21.0
15	—»—	0.60— 1.14 mM P/ 1 whole blood	0 — 2.96
16	—»—	0.5 — 1.9 m. eq P/1 red cells	0.31— 1.18
13	—»—	0.89 mM P/1 whole blood	1.2
14	Berenblum & Chain (1938)	59.4/9.2 μg P/ml/red cells	5.9/0.9
7	Gomori's modification of Fiske & Subbarow (1925)	125 and 173 μM P/100 ml red cells	3.9 and 5.4

Overall range: 0—21 mg/100 ml P

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ably contributed to some of the high values. By observing precautions to minimize hydrolysis during the procedure, Buell (1923) called attention to the possible absence of inorganic P in human erythrocytes.

This paper presents the concentrations of inorganic P found in erythrocytes from 30 healthy, adult individuals and discusses Berenblum & Chain's method for determination of inorganic phosphate (1938) as modified by Martin & Doty (1949).

MATERIALS AND METHODS

Blood sampling.

Heparinized blood obtained by venipuncture was immediately cooled in tubes kept in ice water and centrifuged for 20 minutes at 2500 r. p. m. in a cold room (0—4° C). Plasma and "buffy layer" were discarded. Two milliliters of the packed red cells were transferred to a 25 ml volumetric flask containing 5 ml ice-cold, 20 per cent trichloracetic acid (TCA). The pipet was rinsed in 10 ml ice-cold distilled water, the rinsings transferred to the flask, and volume made up with the cold TCA-solution. In some instances 5 ml of packed red cells and 50 ml flasks were used. Following vigorous shaking with glass beads for one minute, the flasks were left five minutes before filtration.

The centrifuge available in the cold room limited the rotational speed to 2500 r. p. m. (1450 g). By dye-dilution technique, utilizing Evans blue dye (T-1824), it was found that under the conditions used, the packed red cells analyzed for phosphate contained between 10 and 13 per cent trapped plasma.

In five instances packed red cells were washed twice with equal amounts of ice-cold, isotonic saline in a cold room (0—4° C). The second washing was centrifuged for 20 minutes at 2500 r. p. m. to obtain a degree of packing comparable to the packed, unwashed cells.

Determination of inorganic phosphate.

Inorganic phosphate was determined by Berenblum & Chain s method adjusted as follows:

Reagents.

20 % NaOH
1 % paranitrophenol in abs. ethanol (indicator
5 N H_2SO_4
Isobutanol-benzene, equal amounts
5 % ammonium molybdate
Conc. H_2SO_4/96 % ethanol: 32/968 (V/V)
Stannous chloride stock solution: 10 g $SnCl_2 \cdot 2H_2O$ made up to 25 ml with conc. HCl. [illegible] freshly prepared 1 : 200 dilution of stock sol[illegible] distilled water was used in analysis.

Procedure.

Five milliliters of TCA-filtrate in glass-sto[illegible] pered tubes were neutralized with sodium hydroxi[illegible] and then made weakly acid (indicator colorles[illegible] with a few drops of sulfuric acid. Five ml is[illegible] butanol-benzene, 0.5 ml ammonium molybdate a[illegible] 0.5 ml sulfuric acid were added, stoppers appli[illegible] and the tubes shaken for 20 seconds. The phas[illegible] were allowed to separate. One ml of the isobutano[illegible] benzene phase was transferred to photometer tub[illegible] by means of a calibrated Krogh pipet which w[illegible] rinsed twice with sulfuric acid-ethanol. Five [illegible] sulfuric acid-ethanol and 0.5 ml of freshly-ma[illegible] stannous chloride dilution were added and the sol[illegible] tions mixed. After 20 minutes, optical density ([illegible] at 620 mu was read in a Coleman Jr. Spectr[illegible] photometer. Under the conditions outlined, D [illegible] 1,000 corresponded to 44.7 micrograms P.

Discussion of method.

Most methods for determination of P in b[illegible] logical material are based on the formation [illegible] phosphomolybdic acid from inorganic P and sodi[illegible] or ammonium molybdate. The phosphomolybdic a[illegible] is reduced in an acid medium and a proportio[illegible] amount of "molybdenum blue" formed and det[illegible] mined by photometry. Various reducing agents h[illegible] been used and the maximal color development [illegible] tained in from 10 to 30 minutes. During this t[illegible] hydrolysis of organic phosphorus compounds m[illegible] occur and give rise to incorrectly high P_i. Ber[illegible] blum & Chain's method reduces this possibility c[illegible] siderably by extraction of the phosphomolybdic a[illegible] in isobutanol-benzene and removal of the inorga[illegible] phosphate within one minute after addition of [illegible]

its to the sample, thus precluding further in-
ase in P_i due to cleavage of organic P-com-
unds. Ernster, Zetterström & Lindberg (1949)
ve shown that even creatinephosphate can be
ermined by this method. With stannous chloride
ximal color develops in 10 minutes and is stable
at least 12 hours.

Analysis of wet digests of the isobutanol-benzene
ase revealed that no phosphate was present in
cess of the P_i found. Thus no organic P-com-
unds entered the isobutanol-benzene phase from
od filtrate during the extraction. The presence
organic P-compounds in the isobutanol-benzene
ase would seriously impair the reliability of the
thod in radioisotope studies, since radioactive
ay is usually carried out on this phase.

The absence of excess molybdenum in the iso-
anol-benzene phase minimizes possibility of spu-
us reaction which occasionally occur with a
ong reducing agent like stannous chloride. The
thod is less susceptible to changes in acid con-
ntration than most other methods; 1—5 N H_2SO_4
e the same analytical results. It has been claimed
rnster, Zetterström & Lindberg, 1949) that large
ounts of sulfate will disturb the reaction; this
uld not be confirmed. On the other hand NH_3
s found to decrease the sensitivity of the method.

RESULTS

The mean value for P_i in packed, un-
ashed erythrocytes from 30 healthy indi-
duals (3 female, 27 male donors; age 20—
years) was 0.27 mg/100 ml. A statistical
alysis of the data is given in Table II.

The optical densities ranged from .006 to
3, corresponding to 0.07 to 0.43 mg/100
P. These low photometer readings were
finitely below the optimal range for the
strument. Because of this, the values re-
rded have been brought to the nearest
mg/100 ml.

Washing of the erythrocytes was omitted
the series presented in Table II, to avoid
avage of labile phosphate during such a

Table II. *Mg/100 ml inorganic P in packed, unwashed erythrocytes.*

No. of donors	Range	Mean	S.D.	Standard error	Coeff. of var.
30	0.1—0.4	0.27	0.11	0.02	40.6

Table III. *Mg/100 ml P_i in unwashed and washed, packed erythrocytes.*

Sample	Mg/100 ml P_i in packed erythrocytes	
	Unwashed	Washed
1	0.4	0.3
2	0.4	0.3
3	0.4	0.1
4	0.7	0.3
5	0.7	0.2

procedure. P_i was determined in packed, washed erythrocytes in five instances. The results appear in Table III. The P_i in the washed cells was consistently lower than in the unwashed controls.

When blood is allowed to clot at room temperature, a considerable increase in intracorpuscular P_i occurs due to splitting of labile phosphate esters. This subsequently leads to increased serum P_i. Plasma P_i was determined as soon as possible on heparinized, cooled blood. Two aliquots of the same blood without heparin were left to clot for 7 or 24 hours, one in a cold room (0—4°C) where clotting proceeded very slowly, the other at room temperature (22° C). After the allotted time plasma was collected from the cold aliquot and coagulation allowed to proceed at room temperature. At the same time serum from the other aliquot was collected. Determination of P_i was made simultaneously on the two sera upon completion of the plasma clotting. The results are given in Table IV.

Table IV. *Mg/100 ml P_i in plasma and serum. Plasma P_i determined immediately following sampling. Serum P_i after leaving blood samples in cold room (0—4°C) and at room temperature (22°C) for 7 or 24 hours.*

Time lapse before separation of serum from red cells	Plasma P_i	Serum P_i 0—4° C	Serum P_i 22° C
1. 7 hrs.	2.8	3.0	3.2
» 24 »	2.2	2.8	—
2. 7 hrs.	3.1	3.4	3.6
» 24 »	2.8	3.7	3.8

Compared with the immediately obtained plasma P_i values, the results show that even the stay in the cold room caused an increase in serum P_i. This increase was more pronounced in samples left at room temperature.

DISCUSSION

Correct values for P_i in red cells are difficult to obtain. The values for unwashed red cells presented in Table II include the P_i in trapped plasma. Assuming a plasma P_i of 3 mg/100 ml, the 10—13 milliliter of plasma trapped in 100 milliliter of red cells, would contribute 0.30—0.39 mg to the recorded red cell P_i concentrations. This accounts for all the P_i found in red cells.

Whether the P_i found in washed red cells represents true P_i in red cells or derives from hydrolytic cleavage of organic P-esters during the washing procedure is not known. It is evident, however, that the concentration of P_i in red cells must be extremely low.

Rapid performance of the phosphate analysis in the cold can hardly be over-emphasized. The slightest deviation from this rule invariably yielded higher values for P_i than when it was strictly followed.

The precaution of keeping samples co is also of importance for serum P_i dete minations. During the time-lapse require for blood to clot at room temperature, P_i the red cells increases and leads to increase serum P_i as a result of leakage from t red cells.

Correct values for true intracorpuscul P_i are of paramount importance in pho phate turnover-studies with radioactive pho phate. Any cleavage of organic phosphor compounds during the separation procedu will result in falsely high P_i. Consequentl the specific activities obtained, both for and the partly-hydrolysed phosphate este will be misleading.

SUMMARY

1. A discussion of the methods used f determination of inorganic phosphate in r cells is presented. The importance of i mediate cooling of the blood and of centr fuging and protein precipitation in the co is stressed.

2. The mean P_i concentration in packe unwashed erythrocytes from 30 healthy, adu individuals was 0.27 mg/100 ml (± 0.11 the range 0.1—0.4 mg/100 ml. The P_i trapped plasma contributed most of th value.

3. The results are discussed in relati to P^{32} turnover studies in erythrocytes, well as to serum P_i concentrations.

REFERENCES

Berenblum, I. & Chain, E.: XL. An improv method for the colorimetric determination phosphate. Biochem. J. *32*, 295, 1938.

Bloor, W. R.: Methods for the determination phosphoric acid in small amounts of bloo J. Biol. Chem. *36*, 33, 1918.

mskov, C.: Über organische Phosphorverbindungen im Blut. Z. Physiol. Chem. *210,* 67, 1932.

ell, M.: On the phosphorus compounds in normal blood. J. Biol. Chem. *56,* 97, 1923.

nster, L., Zetterström, R. & Lindberg, O.: A method for the determination of tracer phosphate in biological material. Acta Chem. Scandinav. *4,* 942, 1949.

ske, C. H. & Subbarow, Y.: The colorimetric determination of phosphorus. J. Biol. Chem. *66,* 375, 1925.

abrio, B. W., Donahue, D. M. & Finch, C. A.: Erythrocyte preservation. V. Relationship between chemical changes and viability of stored blood treated with adenosine. J. Clin. Investigation *34,* 1509, 1955.

omori, G.: A modification of the colorimetric phosphorus determination for use with the photoelectric colorimeter. J. Lab. Clin. Med. *27,* 955, 1942.

uest, G. M. & Rapoport, S.: Organic acid-soluble phosphorus compounds of the blood. Physiol. Rev. *21,* 410, 1941.

alpern, L.: The transfer of inorganic phosphorus across the red blood cell membrane. J. Biol. Chem. *114,* 747, 1936.

Kay, H. D. & Byron, F. B.: Blood phosphorus in health and disease, I. The distribution of phosphorus in human blood in health. Brit. J. Exper. Path. *8,* 240, 1927.

Martin, J. B. & Doty, D. M.: Determination of inorganic phosphate. Modification of isobutyl-alcohol procedure. Anal. Chem. *21,* 965, 1949.

Mueller, C. B. & Hastings, A. B.: Glycolysis and phosphate fractions of red blood cells. J. Biol. Chem. *189,* 881, 1951.

Prankerd, T. A. J. & Altman, K. I.: A study of the metabolism of phosphorus in mammalian red cells. Biochem. J. *58,* 622, 1954.

Solomon, R. Z., Hald, P. & Peters, J. P.: The state of the inorganic components of human red blood cells. J. Biol. Chem. *132,* 723, 1940.

Snyder, R. & Katzenelbogen, S.: The distribution of sodium, potassium, calcium, magnesium, inorganic phosphorus, and chloride between the blood serum and cells of normal individuals. J. Biol. Chem. *143,* 233, 1942.

Warweg, E. & Stearns, G.: Studies of phosphorus of blood. I. The partition of phosphorus in whole blood and serum, the serum calcium and plasma phosphatase from birth to maturity. J. Biol. Chem. *102,* 749, 1933.

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PHOSPHATE UPTAKE AND GLYCOLYSIS IN HUMAN ERYTHROCYTES HEATED IN VITRO[1]

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INTRODUCTION

Severe burns in humans and animals, as well as *in vitro* heating of blood, cause fragmentation, microspherocytosis, increased osmotic and mechanical fragilities of the red cells (Brown 1946; Shen, Ham & Fleming, 1943; Ham, Shen, Fleming & Castle, 1948; Isaacs, Brock & Minot, 1925; Ponder, 1949; Schubothe & Gross, 1953; Cooray & De, 1949) and a concomitant loss of potassium (McLean, Moritz & Roos, 1947). There is a marked reduction in oxygen consumption and anaerobic glycolysis in nucleated (avian) erythrocytes after prolonged heating (Hunter & Pahigan, 1940). *In vivo* the damaged red cells disappear rapidly from the circulation as a result of hemolysis (Brown, 1946; Ham *et al.*, 1948; Moore, Peacock, Blake'y & Cope, 1946; Jones, Alpen & Davis, 1956).

Previous workers have found temperatures ranging from 48° to 52° C as the critical level for red cell damage following he exposure *in vitro*.

In the present study human blood wa heated *in vitro* for three minutes at tempera tures ranging from 48° to 55° C to evaluat the effect of heat on uptake of radiopho phate by the red cells, and the distributio of intracorpuscular acid-soluble phosphoru compounds. Some observations on glucos utilization by heated red cells are include

METHODS

Freshly drawn, heparinized venous blood sampl from healthy subjects were divided in two equ portions. One was heated for three minutes at 48° 51°, 53°, or 55° C, the other observed as control. A samples were kept in ice water except during th heating procedure. Rapid heating of the exper mental sample to 1° C below the final temperatur was achieved with vigorous stirring in a sma waterbath with a temperature approximately 10° above the final temperature for the sample. Upo transfer to a constant temperature waterbath, th desired temperature was reached quickly and mai tained (± 0.02° C) for three minutes. The samp was then immersed in ice water and cooled to th temperature of the control.

Incubation with P^{32}*, 15—30 microcuries p milliliter of blood, was carried out under consta

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* Obtained from Abbott Laboratories, O Ridge, Tenn.

ıking for four hours at 37° C in atmospheric air.) attempts were made to ensure sterility.

P³² uptake: Aliquots for assay of radioactivity l hematocrit determinations were obtained at the ginning and at hourly intervals during the in- ɔation. Counting of radioactivity was performed a well-type *beta* counter. Enough counts to secure probable counting error of less than 1 per cent re obtained.

Although the white cells are actively metabolising elements of blood, it has been shown that their contribution to the total P^{32} uptake in blood cells is negligible (Gourley & Gemmill 1950). In the present study the radioactivity of white cells and platelets has been disregarded and the difference between whole blood and plasma radioactivity considered as red cell radioactivity.

Calculation: Incorporation of P^{32} into red cells in the hourly aliquots was calculated and related to whole blood radioactivity as follows:

$$\frac{\text{Counts/min./ml whole blood} - \text{counts/min./ml plasma} \times \frac{(100 - \text{Hct.})}{100}}{\text{Counts/min./ml whole blood} \times \frac{\text{Hct.}}{100}} \times 100 \times 0.1$$

ıere the whole blood radioactivity represents the ɛan of the activities at start and completion of ɛ incubation. These were in good agreement. Hct. Mean of uncorrected hematocrits.

This expression gives the uptake per 0.1 milliɛr of red cells as percentage of the radioactivity 1 milliliter of whole blod. A linear relationship tween uptake and time could not be established. owever, the uptake values expressed in the above ɑnner offered a convenient basis for comparison the control group with the groups of heated ɔod.

Phosphate fractionation: Aliquots for deterɪnation of intracorpuscular acid-soluble phosɔrus compounds were taken out following cooling the heated sample. The aliquots were centrifuged r 20 minutes at 2500 r.p.m. in a cold room —4° C), plasma and "buffer layer" discarded. ıe phosphorus compounds in trichloracetic acid ɪtracts of the packed red cells were frationated determination of P_i (inorganic phosphate), P_i ɔerated after ten minutes and three hours hydrosis with 1 N HCl on a boiling waterbath and in ɛt-ashed extracts. P_i was determined by the meɔd of Berenblum & Chain (1938).

Glucose determinations: Blood glucose was deɾmined by the anthrone method (Zipp & Waldo, 52) after precipitation of the proteins with ı(OH)₂($ZnSO_4$ + NaOH) on a boiling waterth.

RESULTS

P^{32} uptake in normal human erythrocytes. The mean P^{32} uptake after four hours of incubation of blood from 35 healthy donors was 15.7 per cent per 0.1 milliliter of packed red cells (standard deviation 1.0 per cent, standard error 0.17 per cent). The mean, hourly uptake values are plotted against time in Fig. 1 as per cent of the mean four-hour uptake (15.7 per cent).

The results spread over a fairly wide range, which, however, becomes smaller with increasing time of incubation. At four hours the results exhibit a symmetrical frequency distribution with 74 per cent of obtained values within $\pm$ 1 S.D. (standard deviation) and 91 per cent within $\pm$2 S.D.

Plotting of four-hour uptake against hematocrits revealed no correlation between these two variables within the hematocrit range, 39.5 to 51.6 per cent.

P^{32} uptake in erythocytes from heated versus unheated blood. Groups of five or six individual blood samples were heated for three minutes at 48°, 51°, 53° and 55° C

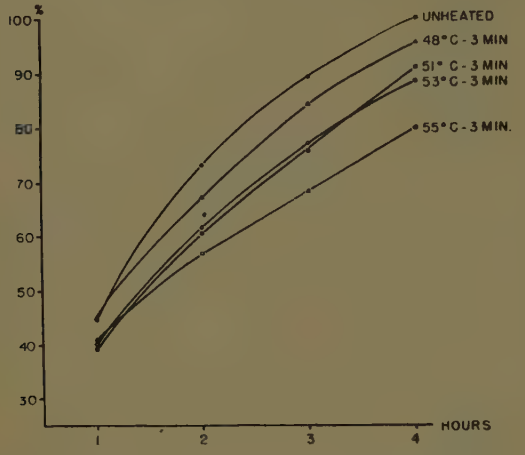

Fig. 1. Uptake of P^{32} during 4 hours of incubation at 37°C by normal erythrocytes and by erythrocytes from blood heated for 3 minutes at 48,° 51°, 53° or 55° C. For unheated blood: 100 per cent = Mean normal 4-hour uptake (35 experiments). For heated blood: 100 per cent = 4-hour uptake in the unheated control for each sample. Means of 5 or 6 experiments in each group plotted.

respectively, and incubated simultaneously with unheated control samples of the same blood. The phosphate uptake values obtained are recorded as percentage of the four-hour uptake in the control. The means for each group are plotted in Fig. 1 where the curve best fitting the four points actually determined has been drawn.

It is apparent that the incorporation of P^{32} into the erythrocytes of heated blood is reduced compared to normal. Except for the intersection of the curves for 51° and 53° C, the reduction increases with rising temperature. Statistical analysis reveals that the reduction is not significant in the 48° C group, although it was consistently found, or for the one-hour uptake in the remaining three groups ($p > 0.05$). The other values are significantly lower than normal ($p < 0.01$).

Heating of plasma only did not produc this effect, whereas separately heated, packe red cells showed a reduction in P^{32} uptak upon resuspension in plasma and incubatio of the same magnitude as red cells fro heated whole blood.

Addition of adenosine (six incubations to a final concentration of 2500 micromole per 100 milliliters of red cells (Gabrio, Her nessy, Thomassen & Finch, 1955) failed t restore the P^{32} uptake of heated red cells t normal. On the contrary, in three of the si incubations with adenosine, a further dro in P^{32} uptake occurred.

Acid-soluble phosphorus compounds i heated and unheated red cells. In Table I th results of phosphate fractionation in trichlor acetic acid extracts of heated and unheate packed red cells are given. The recorde values represent net P_i in the fractions.

The heating did not alter the distributio pattern of the acid-soluble phosphorus fra tions in red cells to any great extent.

Glucose utilization and esterification of P Blood glucose determinations and assay o radioactive P_i and total acid-soluble P in re cells were performed at the onset and upo completion of the four-hour incubation t control blood and blood heated for thre minutes at 55° C. The results appear i Table II.

It is evident that glucose was utilized an radioactive orthophosphate esterified in th red cells subjected to a temperature of 55° C although at a reduced rate, in three of th five experiments.

In one instance the morphology of re cells after heating for three minutes at 55° was studied in smears stained with Wright stain. The heating produced a fall in hem

ıble I. *Phosphate-fractionation in TCA-extracts of packed red cells from unheated and ıted blood. Net P in the fractions is recorded as microgram P per milliliter of packed red ls. For control red cells means — S.D., and range (in parenthesis) are listed. For heated red cells means and range (in parenthesis) are given.*

Treatment (No. of experiments)	Pi	10 min. hydrolyzable P	3 hrs. hydrolyzable P	P not hydrolyzed in 3 hrs.	Total acidsol. P
Unheated controls (30)	2.7—1.1	68— 6	121— 19	268— 39	460— 53
	(1—4)	(52—82)	(74—151)	(195—338)	(356—543)
48°C—3 min. (5)	1.6	64	108	245	433
	(1—3)	(60—71)	(70—126)	(212—280)	(382—468)
51°C—3 min. (6)	1.7	72	114	235	425
	(1—3)	(63—93)	(87—154)	(151—294)	(300—482)
53°C—3 min. (6)	1.7	68	122	272	442
	(1—3)	(59—95)	(74—154)	(215—345)	(356—482)
55°C—3 min. (6)	2	68	95	204	362
	(1—3)	(48—97)	(65—119)	(157—273)	(271—459)

ıble 2. A: *Glucose Utilization during 4 hours incubation at 37°C, and* B: *Specific activity ²/P as counts/min./microgram P) of organic acid-soluble phosphorus in red cells at the inning (0 hrs.) and end (4 hrs.) of 4 hours incubation of control blood and blood heated for 3 minutes at 55°C.*

xperiment	A. Glucose Utilization				B. Spec. act. (P^{32}/P) in organic acid-soluble phosphorus			
	Mg/hr./100 ml blood		Mg/hr./ml red cells		Control		Heated	
	Control	Heated	Control	Heated	0 hrs.	4 hrs.	0 hrs.	4 hrs.
1	10.8	8.5	0.27	0.27	—	—	—	—
2	10.3	8.8	0.23	0.24	210	1190	152	940
3	13.8	9.0	0.30	0.21	148	1334	364	1221
4	14.0	6.5	0.30	0.15	36	179	86	331
5	15.0	9.8	0.31	0.25	413	1256	292	817

rit from 42.7 per cent to 33.0 per cent. e red cells showed extensive budding ir- ular fragmentation, anisocytosis with ısely stained microcytes and pale macro- ic "ghosts". A number of the red cells ɔeared normal, however, retaining their coid shape and staining normally. No ıntitation of these cells was made. The erved morphological changes remained entially the same during four hours of ubation at 37° C.

DISCUSSION

The observed reduction of P^{32} uptake in red cells from heated blood is probably caused by a direct thermal effect on the red cells. The greatest changes after heat exposure presumably occur in the red cells that hemolyse. These changes escape detection in the present study.

The red cells that do not hemolyse, as represented by the packed mass in the hematocrit tubes, retain, to a considerable extent,

their ability to take up radiophosphate from the medium and to incorporate P^{32} into phosphate esters.

It has been shown (Isaacs *et al.*, 1925) that immature red cells less readily undergo morphological changes than mature red cells at 55° C. Considering the small number of immature cells in a normal red cell population, it appears unlikely that they alone could be responsible for the observed P^{32} uptake.

It might be expected that subjection of red cells to heat would be inimical to their heat labile enzymes. Complete heat inactivation of enzyme activity with "freezing" of intracorpuscular phosphate fractions, and passive diffusion of P^{32} through an abnormally permeable red cell membrane, might explain the observed pattern of cellular phosphates and P^{32} uptake.

However, glucose utilization, although reduced, proceeds, and inorganic phosphate is esterified in red cells from heated blood. The observations suggest that glycolytic activity and phosphate uptake are interdependent processes also in the heated red cells and that the reduction in P^{32} uptake in these cells is an indication of decreased glycolytic activity.

Reduction of glycolytic metabolism and a corresponding decline in energy available for preservation of red cell integrity, may account for the earlier observations on morphological and viability changes in heated red cells.

As red cell "ghosts" are unable to utilize glucose (Lionetti, Rees, Healey, Walker & Gibson, 1956), the observed glucose utilization must be attributed to red cells with some extent of retained glycolytic enzyme activity. Despite the presence of glycolytic activity post heating, irreversible damage is apparently inflicted upon the red cells. Adenosin addition, in amounts known to reverse th "storage lesion" of red cells (Gabrio *et al* 1955), failed to restore the phosphate uptak by heated red cells to normal. This points t heat inactivation of the nucleoside phos phorylase as one possible consequence o heating.

Whether the observed glycolytic activit corresponds to preserved viability of som of the heated red cells is not known. Post transfusion survival studies on such cell must provide the ultimate answer concern ing their viabiliay.

SUMMARY

1. Human blood was heated for thre minutes at 48°, 51°, 53°, and 55° C.

2. P^{32} uptake in the red cells during fou hours of incubation at 37° C was determine in heated and control blood. Intracorpuscula acid-soluble phosphates were fractionated b acid hydrolysis.

3. The figures for P^{32} uptake in the re cells after two, three and four hours of i cubation were lower than normal in eac group of heated blood samples. The diffe ence was not statistically significant for th group heated at 48° C. Adenosine additio did not restore to normal the reduced P uptake in heated red cells. The pattern of i tracorpuscular acid-soluble phosphate com pounds was essentially unchanged aft heating.

4. In five experiments red cells fro blood heated for three minutes at 55° utilized glucose and incorporated P^{32} in organic acid-soluble P-compounds, althou at a reduced rate compared to normal.

5. The results indicate that phosphate up-
te and glycolytic activity are closely related
o in heated red cells.

REFERENCES

renblum, I. & Chain, E.: XL. An improved method for the colorimetric determination of phosphate. Biochem. J. *32,* 295, 1938.

own, A.: Morphological changes in the red cells in relation to severe burns. J. Path. Bacteriol. *58,* 367, 1946.

oray, G. H. & De, S. N.: Red cells changes in burns and acute anhydraemia. J. Path. Bacteriol. *61,* 541, 1949.

brio, B. W., Hennessy, M., Thomasson, J. & Finch, C. A.: Erythrocyte preservation. IV. *In vitro* reversibility of the storage lesion. J. Biol. Chem. *215,* 357, 1955.

urley, D. R. H. & Gemmill, C. L.: The effect of temperature upon the uptake of radioactive phosphate by human erythrocytes *in vitro*. J. Cell. Comp. Physiol. *35,* 341, 1950.

m, T. H., Shen, S. C., Fleming, E. M. & Castle, W. B.: Studies on the destruction of red blood cells. IV. Thermal injury: Action of heat in causing increased spheroidicity, osmotic and mechanical fragilities and hemolysis of erythrocytes; observations on the mechanisms of destruction of such erythrocytes in dogs and in a patient with a fatal thermal burn. Blood *3,* 373, 1948.

nter, F. R., & Pahigan, V.: The effect of temperature on cell permeability and on cell respiration. J. Cell. Comp. Physiol. *15,* 387, 1940.

Isaacs, R., Brock, B. & Minot, G.: The resistance of immature erythrocytes to heat. J. Clin. Investigation *1,* 425, 1925.

Jones, D. M., Alpen, E. L. & Davis, A. K.: Acute erythrocyte destruction in severe thermal injury. Am. J. Physiol. *184,* 147, 1956.

Lionetti, F., Rees, S. B., Healey, W. A., Walker, B. S. & Gibson, J. G.: II. The effect of adenosine upon the esterification of phosphate by erythrocyte ghosts. J. Biochem. *220,* 467, 1956.

McLean, R., Moritz, A. R. & Roos, A.: Studies of thermal injury. VI. Hyperpotassemia caused by cutaneous exposure to excessive heat. J. Clin. Investigation *26,* 497, 1947.

Moore, F. D., Peacock, W. C., Blakely, E. & Cope, E.: Anemia of thermal burns. Ann. Surg. *124,* 811, 1946.

Ponder, E.: The tonicity-volume relations for human red cells subjected to the action of heat, with special reference to prolytic K loss. J. Gen. Physiol. *32,* 399, 1949.

Schubothe, H. & Gross, F.: Wärmeveränderungen roter Blutkörperchen. Schweiz. med. Wchnschr. *83,* 1048, 1953.

Shen, S. C., Ham, T. H. & Fleming, E. M.: Studies on the destruction of red blood cells. III. Mechanisms and complications of hemoglobinuria in patients with thermal burns: Spherocytosis and increased osmotic fragility ofred b lood cells. New England J. Med. *229,* 701, 1943.

Zipp, R. E. & Waldo, A. L.: Spectrophotometric analysis of carbohydrates and study of anthrone reagent. J. Lab. Clin. Med. *39,* 497, 1952.

Scandinav. J. Clin. & Lab. Investigation 50—53, 9, 1957.

THE VITAMIN B_{12}-BINDING PRINCIPLE OF HUMAN GASTRIC JUICE: INFLUENCE OF pH ON THE BACTERIAL ADSORPTION OF FREE AND BOUND B_{12}

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The capacity of certain bacteria to adsorb free, but not bound, vitamin B_{12} has been utilized for the assay of vitamin B_{12}-binding power. In a recent study on gastric juice (Gräsbeck, 1956), the results of this method were found to correlate well with those of a dialysis method, provided the pH was close to neutral. However, if the pH was off this optimum, certain changes in the bacterial adsorption of the vitamin were observed, and a more detailed study of this phenomenon is reported in the following.

MATERIAL AND METHODS

The collection of gastric juice was done as in the previously quoted report (Gräsbeck, 1956), with the exception that the juice was obtained from patients with no evidence of gastric disorder. Some juices were used acid a short time after collection, others were first pepsin-inactivated by alkalinization and then adjusted to pH 7. To the juice was added radioactive Co^{60}-labeled vitamin B_{12} ("Merck", specific activity 160 μc/mg) and the mixture was diluted with water in such a manner that 1 ml of the final dilution contained 50 mμg of the radio-vitamin, most of which was in bound form (*i. e.*, was not taken up by a bacterial suspension at pH 7). An aqueous solution of 50 mμg radio-B_{12} per ml was used as control. Before further use, the solutions were allowed to stand for 20 min.

Bacterial Suspensions. Lactobacillus leichmanni ATCC 4797 suspensions were prepared as in Gräsbeck (1956). Two *Escherichia coli* strains E 35 (Hoff-Jørgensen, Skouby & Gad Andresen, 1952) and K7b (Levanto, 1954) were grown for three days in the medium II described by Hoff-Jørgensen, Skouby & Gad Andresen (1952), and the cells were harvested by centrifugation. The harvested bacteria were washed three times with ice-cold physiological saline before the final suspension was made. The final saline suspension gave a reading of 450—500 with a # 54 filter in a Klett-Summerson colorimeter with a water blank.

Buffers. The desired pH was produced by adding 0.1 N NaOH to a mixture of 0.04 moles of both acetic, boric and o-phosphoric acid, which had been diluted to a volume of one liter. Irrespective of pH, this buffer was considered to be 0.12 molar. For pH 1, 0.12 N HCl was used. By adding water to these ready-made buffers, new buffers of weaker molarity could be produced. Stronger buffers were made in an analogous way.

The usual technique consisted of preparing two series of test-tubes containing 8 ml buffer of pH 1—8. To one series was added 1 ml of the gastric juice — radiovitamin B_{12} solution, to the other series 1 ml of the aqueous solution of radio-B_{12}. The tubes were sealed with paraffin film ("Parafilm") and were inverted a few times, then one ml of the bacterial suspension was added and the tubes were inverted again. If undiluted buffer was used, the resulting molarity was considered to be = 0.1. After addition of *L. leichmannii* the tubes were centrifuged after 30 min., whereas the *E. coli* cells were given 60 min. for B_{12}-adsorption. Centrifugation was done for 20 min. at 2000 RPM. Afterwards the clear supernatants were quickly decanted off; they were usually discarded, but were saved in a few instances for further study.

ble. *Experiment to elucidate whether leichmannii resting cells detach bound B_{12} m combination with binding principle, or dsorb the whole binding principle — B_{12} complex at low pH.*

mple + 50 mμg radio-B_{12} (= 650±15 CPM)	Radioactivity (CPM) recovered in resting cells after centrifugation
binder, pH 7	660±20
binder, pH 1	540±10*
ıtralized supernatant of eceding + new radio-B_{12}	650±3
tric juice, pH 7.....	45±5
tric juice, pH 1.....	510±10*
ıtralized supernatant of eceding + new radio-B_{12}	40±10

N.B. slight inhibition of radio-B_{12} adsorption at pH 1.

n contrast to the earlier technique, the recovered teria were not transferred to planchets, but were nted directly in the centrifuge tubes in a sc'n-tion counter. A tube with 50 mμg radiovitamin lry form on the bottom was used as standard.

RESULTS

n complete accordance with the findings Davis (1953), it was observed that *L. hmannii* took up B_{12} inefficiently at high 's (above 7). At low pH's the uptake was icient, approaching 100 per cent in the ole acid range. In some experiments slight ibition at pH 1 was observed. Molarities ve 0.1 were found to inhibit the percent-uptake with increasing molarity.

The uptake of B_{12} by the *E. coli* strains s never so efficient as that by *L. leich-nnii*. In the best range, pH 6—8 (higher 's not investigated), the uptake was about per cent. The uptake declined rapidly at er pH's. The exact pH range of the de-

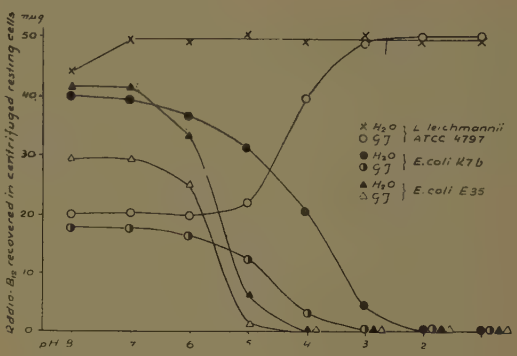

Fig. 1. The influence of pH on the adsorption of free and bound B_{12} by different bacteria. Molarity of the buffers = 0.1. GJ = gastric juice (binding principle present).

cline varied somewhat in different experiments. Thus at an early stage the *E. coli* strains were found to be unsuitable for studying B_{12}-binding at low pH's.

The results suggest a difference in the mechanism of B_{12}-adsorption between *L. lecihmannii* and *E. coli*.

Uptake of "bound B_{12}" at low pH

That vitamin B_{12} is still bound to gastric juice is evident, because bound vitamin cannot be dialyzed out at low pH's, provided the binding principle is not broken down by autodigestion. The binding capacity is stable to exposure to pH 1—12 for at least 30 min. (Gräsbeck, 1956).

As is seen from Fig. 1, it was observed that "bound B_{12}", which was unvailable to *L. leichmannii* at pH 5—7, was taken up by the bacterial suspension at lower pH's. This phenomenon was studied numerous times in the presence of different amounts of binder. Even though the binding substance was present in amounts several times sufficient to make the vitamin completely unvailable to the bacteria at neutral pH, the bacteria were able to take up considerable amounts of the

vitamin at low pH's. This was found to be true at molarities up to 0.15. The uptake of the bound vitamin by *L. leichmannii* at low pH's could be demonstrated with the gastric juices, irrespective of whether the vitamin had been added to acid juice or to previously alkalinized neutral juice.

Similar studies were made with the *E. coli* suspensions. As is seen from Fig. 1, the phenomenon described could not be reproduced with these bacteria.

Nature of the uptake of bound B_{12} at low pH

Since it was possible that at low pH the bacterial suspension either detached the bound vitamin or adsorbed the whole binding principle — vitamin B_{12} complex, the following experiment was performed: A dilution of gastric juice and radioactive B_{12}, in which most of the vitamin was unvailable to the bacteria at pH 7, was treated with the *L. leichmannii* suspension at pH 1, and the bulk of the radioactivity was recovered in the bacteria after centrifugation. The supernatant was neutralized, new radiovitamin was added, and the binding power was assayed at pH 7. As is seen from the table, the binding property at pH 7 persisted, and was of the same magnitude as before acidification. Thus the bacterial suspension had detached the bound vitamin and not adsorbed the whole complex.

DISCUSSION

The "detachment phenomenon at low pH" deserves attention from some points of view: Firstly, it may give a clue to the chemical nature of the binding phenomenon. Secondly, in order to obtain comparable results in B_{12} binding assay with the microbial adsorptic inhibition method and the dialysis metho one should operate close to neutral pH. Th is also desirable because autodigestion by th "gastric protease" is largely inhibited pH 7. Thirdly, the phenomenon may hav some bearing on the observation that Castle intrinsic factor lacks hematopoietic activit at low pH (Castle, Heath, Strauss & Heinl 1937, Cox, Ross & Ungley, cit. by Ungle 1955).

It remains an open question whether th detachment phenomenon is due to an i creased affinity for B_{12} in *L. leichmannii* to a decreased affinity for B_{12} in the bindi principle.

SUMMARY

Lactobacillus leichmannii resting cells we found to adsorb about 100 per cent free B at pH 1—7, at higher pH's the uptake dim ished. Two *Escherichia coli* strains adsorb about 80 per cent free B_{12} at pH 6— whereas the adsorption was strongly inhi ited by low pH's. With all the studied ba teria gastric juice inhibited the uptake B_{12} at neutral pH; at low pH the bound vit min was detached from the binding pri ciple and taken up by the *L. leichmannii* re ing cells. The latter phenomenon could n be demonstrated with *E. coli*.

ACKNOWLEDGEMENTS

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REFERENCES

tle, W. B., Heath, C. W., Strauss, M. B. & Heinle, R. W.: Observations on the etiologic relationship of achylia gastrica to pernicious anemia. VI. Site of interaction of food (extrinsic) and gastric (intrinsic) factors; failure of *in vitro* incubation to produce thermostable hematopoietic principle. Am. J. M. Sc. *194*, 618, 1937.

vis, R. L.: Biochemical studies of synthesis and uptake of vit. B_{12} by rat and bacteria. Dissertation Johns Hopkins Univ. School of Hygiene and Public Health, Baltimore, 1953.

Gräsbeck, R.: Studies on the vitamin B_{12}-binding principle and other biocolloids of human gastric juice. Acta med. Scandinav. Suppl. 314, 1956.

Hoff-Jørgensen, E., Skouby, E. P. & Gad Andresen, J.: Mikrobiologisk standardisering og klinisk effekt af koncentrerede præparater af "intrinsic factor". Nord. Med. *48*, 1754, 1952.

Levanto, A.: A study of gastric *Escherichia* strains with special reference to pernicious anemia. Ann. med. int. Fenniae Suppl. 20, 1954.

Ungley, C. C.: The chemotherapeutic action of vitamin B_{12}. Vitamins and Hormones *13*, 137, 1955.

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AMINO ACIDS IN BLOOD PLASMA AND URINE DURING PREGNANCY

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It is well known that during normal pregnancy there is an increased excretion of most of the amino acids with the urine. Honda (1923) was the first to report increased amino acid excretion during pregnancy, and later Voge (1929) and Kapeller-Adler (1933) confirmed this for histidine and proposed the determination of histidine in the urine as a pregnancy test. The studies of Lawrie (1947) indicated a rise in tyrosine excretion during pregnancy. Using microbiological methods Wallraff, Brodie & Borden (1950) demonstrated a significantly increased excretion of histidine, tyrosine, serine, arginine, phenylalanine, threonine and tryptophane, whereas the excretion of aspartic acid and lysine approached a significant level. Sheft & Oldham (1952) supplemented these investigations, finding increased excretion of methionine during the second half of pregnancy. Besides the amino acids mentioned, Miller, Ruttinger & Macy (1954) have found an increased excretion of valine, leucine and isoleucine during pregnancy.

Page, Glendering, Digman & Harper (1954) have found increased histidine clearance during pregnancy after intravenous infusion of histidine.

As simultaneous quantitative analyses of amino acids of plasma and urine in preg nancy have apparently not been made, apar from the investigations just mentioned, was found desirable to supplement previou research with such determinations.

The present study has been confined t three healthy women in the 3rd—4th monti of pregnancy and three normal non-pregnan women. Our investigations show that a this time in pregnancy the clearances of considerable number of amino acids are in creased (α-aminobutyric acid, arginine, glu tamic acid, glycine, histidine, leucine, lysine methionine, serine, threonine and tyrosine) The plasma levels of most of these amin acids were found to be moderately decrease during pregnancy.

MATERIALS AND METHODS

Amino acid clearance determinations were ca ried out on 6 women, cf. Table I. The *norm group* (1, 2, 3) consisted of non-pregnant wom with minor gynecological complaints. In none the cases were essential pathological changes o served. The *pregnant women* (4, 5, 6) were the 3rd—4th month of pregnancy. They present no symptoms of disease at the time of the clea ance determinations. During the last week befo the clearance determinations both groups we given the normal diet of the hospital and th were kept in bed with permission to visit t lavatory and leave the bed to wash.

Table I. *Data for subjects investigated.*

Name	Journal No.	Age years	Height cm	Weight kg	Remarks
E.K.S.	Gyn. 870/56	32	162	66.0	Sterilitas
I.M.G.	Gyn. 455/56	26	160	52.0	Erosio colli uteri
G.E.A.	Gyn. 597/56	34	162	53.5	Obs. pro salpingo-oophoritis chron.
A.M.J.	191/56	35	169	63.0	Graviditas m. 3. Emesis, stopped 2 weeks before clearance determination.
C.S.	I 342/56	35	161	57.6	Graviditas m. 3. Obs. pro abortus imm. No symptoms the last 10 days before clearance determination.
K.T.	H 350/56	32	162	69.5	Graviditas m. 4. Obs. pro fibroma uteri.

Collection and analysis of samples. At 7.30 a.m. fasting person drinks 500 ml of water. At 8 ock a.m. the urinary bladder is emptied by heterization and the catheter is laid *à demeure* closed. At 12 o'clock the bladder is emptied in, and the urine is collected. The bladder is n washed with about 50 ml distilled water which ollected separately. At 10 a.m. a blood specimen drawn from the cubital vein. For each sample 20 ml blood 5 mg heparine is added. The diuresis the clearance period was computed by means nitrogen determinations on urine and wash er.

The ion exchange chromatography method (preation of the resins and buffers and the elution nique) of Moore & Stein (1954a) was used the determination of amino acids. In some liminary experiments we tried different degrees cross-linkings of the cation exchange resin wex AG 50, but finally used a mixture of equal ts of the 5 per cent and the 4 per cent crossed resins X5 and X4. Fractions of about 1 ml obtained by a Hössli drop-counting fraction ector. The ninhydrin reagent used is stored a black glass bottle under N_2-atmosphere and prepared according to the method described by ore & Stein (1954b). For each fraction 0.75 ml ydrin reagent is used. Before readings in the kman spectrophotometer (proline at 440 mμ, other amino acids at 570 mμ) 5 ml ethanol-er = 50 : 50 were added. In the range of glycine-ine and of histidine in the urine chromatograms as necessary to prepare dilutions with citrate ers in order to keep within the range of proportionality between amino acid concentration and color development.

Between 15 and 20 ml acidified urine were usually used for each chromatogram, Stein (1953). The blood samples were centrifuged, and the resulting plasma was treated with an equal volume of 10 per cent trichloracetic acid. The mixture was centrifuged and filtered, and 15 ml of the filtrate were treated by a freeze-drying procedure in order to remove the main part of the trichloracetic acid. The dried filtrate weighing about 200 mg was dissolved in 5 ml 0.01 N HCl and added to the column. Quantitative analyses of known mixtures of amino acids, treated with trichloracetic acid as above indicated, showed that this treatment did not influence the composition of the solution.

The chromatographically separated amino acids were identified through paper chromatography on desalted fractions. The desalting procedure was for the acid and the neutral amino acids the method described by Stein (1953), except that we used Amberlite IRA 400 instead of Dowex 2. For the basic amino acids we used Dowex 50—X12 and followed the procedure of Drèze, Moore & Bigwood (1954). The desalted effluents were collected and evaporated in vacuum desiccators. The paper chromatography was performed as ascending chromatography on Whatman paper, No. 1. The following systems were used. For acid and neutral amino acids: (1) sec. butanol: formic acid: water = 75 : 13 : 12, (2) n-butanol saturated with 2 N NH_4OH, (3) pyridine: water = 65 : 35, and (4) pyridine: acetic acid: water = 50 : 35 : 15. For the basic amino acids were used (4), (5) phenol:

Table II. *Color yield from amino acids on molar basis relative to leucine.*

Amino acid	Color yields This study	M. & S.	Amino acid	Color yields This study	M. & S.
Taurine	0.97	0.88	Cystine (half)	0.55	0.55
Aspartic acid	0.95	0.94	Methionine	0.99	1.02
Threonine	0.96	0.94	Isoleucine	1.00	1.00
Serine	0.98	0.95	Leucine	1.00	1.00
«Asparagine»	0.93	0.95	Tyrosine	0.98	1.00
Proline (440 mμ)	0.239	0.225	Phenylalanine	0.99	1.00
Glutamic acid	1.01	0.99	Ornithine	1.09	1.12
Glycine	0.96	0.95	Lysine	1.07	1.10
Alanine	1.00	0.97	1-CH_3-histidine*)		0.88
α-NH_2-butyric acid	1.01	1.02	Histidine	0.99	1.02
Valine	0.97	0.97	Arginine	1.01	1.01

M. & S. = Moore & Stein (1954 b).
*) Dr. H. H. Tallan, The Rockefeller Institute for Medical Research has kindly given us a small amount 1-methylhistidine, necessary for the identification of the 1-methylhistidine peak. For calculation of this ami acid the color value found by Moore & Stein was used.

Table III. *Recoveries of amino acids from synthetic mixtures.*

Amino acids	Recovery in per cent	S.D.	Amino acids	Recovery in per cent	S.D.
Aspartic acid	101	4	Methionine	95	2
Threonine	102	3	Leucine	101	5
Serine	101	2	Tyrosine	99	1
Proline	98	2	Phenylalanine	98	4
Glutamic acid	99	3	Lysine	99	3
Glycine	101	2	Histidine	98	3
Alanine	101	4	Arginine	96	2

S.D. = standard deviation.

water = 100 : 20 (from phenolum liquidum) with an atmosphere of 2 N NH_4OH, and (6) methanol 67 per cent with an atmosphere of 2 N NH_4OH. These systems were taken from Turba (1954) and Block, Durrum & Zweig (1955). Creatinine was identified by Jaffe's test and arginine confirmed by Sakaguchi's test.

The paper chromatography revealed the peaks containing the following amino acids in the urine chromatograms to be contaminated with traces of ninhydrin positive material, possibly mostly peptides: taurine, threonine, methionine, isoleucine, leucine, tyrosine and phenylalanine. In the glycine peak traces of citrulline were found. The asparagine peak consisted of 3 to 4 components of which asparagine and sarcosine have been identified and this compound is therefore given with quotat marks in the tables. Valine and cystine were separable on the resin used and quantitative det mination of these amino acids was impossible.

The color yields, I_c, obtained and used for different amino acids were close to those repo by Moore & Stein (1954b), cf. Table II. Only the case of taurine was a slight difference fo

The mean volume, a, of the fractions in a p is calculated on the basis of volume determi tions (weight/specific weight) of about every f fraction. Deviations from the standard vol 1.00 ml, are corrected by multiplying the extinction, E, for each peak with a factor f,

$$f = \frac{\text{diluent-vol.} + \text{ninhydrin-vol.} + a}{\text{diluent-vol.} + \text{ninhydrin-vol.} + 1.00}$$

he amount of amino acid in mg in a peak is ılated from the formula

$$\text{mino acid, mg} = \frac{E \times f \times \text{molecular weight}}{\alpha \times I_c \times 1000}$$

hich α is the extinction produced by 1 ml 1 mMol ine solution. The α-value was computed from ral determinations on each ninhydrin solution ared.

he results of recovery experiments on amino mixtures of known composition (5 experits) and on urines with and without addition he known amino acid mixture (3 experiments) summarized in Table III. In general the rery was found to be within 5 per cent of the cted value, except for methionine and arginine 95 ± 2 per cent and 96 ± 2 per cent respecy. In the calculations of the amounts of methioand arginine the values for these amino acids were divided by 0.95 and 0.96 respectively to correct for losses during chromatography. Creatinine was determined in urine and blood plasma according to the method of Borsook (1935).

The clearance for creatinine and the amino acids was calculated according to the formula UV/P and expressed in milliliters per minute per 1.73 sq.M.

RESULTS

The results are given in Tables IV and V. It is seen that the urinary excretion of several of the amino acids is increased during pregnancy. In Table VI the ratio between the average clearance values for the different amino acids during pregnancy and in the non-pregnant state is shown, and the results

Table IV. *Results in non-pregnant women (1, 2, 3).*

Amino acid	Plasma µg/ml				Urinary excretion µg/min.				Renal clearance ml/min. per 1.73 m²			
	1	2	3	Avg.	1	2	3	Avg.	1	2	3	Avg.
rine	7.2	4.6	3.9	5.2	23.8	—	12.3	18.1	3.4	—	3.5	3.5
artic acid	1.4	1.5	1.2	1.4	0.9	0.8	0.8	0.8	0.7	0.6	0.7	0.7
eonine	18.5	12.7	15.8	15.7	12.2	12.3	9.4	11.3	0.7	1.1	0.7	0.8
ne	17.2	15.2	13.9	15.4	30.4	38.4	22.9	30.6	1.8	2.9	1.8	2.2
paragine»*)	26.6	22.6	34.6	27.9	18.9	26.5	22.9	22.8	0.7	1.3	0.7	0.9
ine	20.6	20.4	19.3	20.1	1.8	1.2	2.0	1.7	0.1	0.1	0.1	0.1
tamic acid	5.2	4.0	6.3	5.2	2.2	2.3	2.1	2.2	0.4	0.7	0.4	0.5
cine	43.8	30.9	21.0	31.9	137.5	152.8	90.5	126.9	3.2	5.6	4.8	4.5
ine	20.7	18.7	23.5	21.0	7.7	10.9	7.9	8.8	0.4	0.7	0.4	0.5
H_2-butyric acid	3.6	2.6	1.6	2.6	2.0	3.4	1.7	2.4	0.6	1.5	1.2	1.1
hionine	3.7	3.1	3.4	3.4	4.3	3.6	2.8	3.6	1.2	1.3	0.9	1.1
eucine	7.9	7.4	6.8	7.4	8.0	7.0	12.4	9.1	1.0	1.1	2.0	1.4
cine	10.7	14.4	14.6	13.2	3.3	3.9	4.1	3.8	0.3	0.3	0.3	0.3
osine	8.6	7.1	7.4	7.7	6.5	8.0	7.5	7.3	0.8	1.3	1.1	1.1
nylalanine	7.9	8.0	7.7	7.9	5.5	7.5	4.7	5.9	0.7	1.1	0.7	0.8
ne	22.0	17.5	21.6	20.4	10.2	3.6	4.4	6.1	0.5	0.2	0.2	0.3
idine	9.1	10.2	13.0	10.8	50.3	44.3	52.3	49.0	4.6	4.9	4.5	4.7
H_3-histidine	—	—	3.6	—	5.4	13.6	—	9.5	—	—	—	—
nine	12.5	11.2	7.7	10.5	5.4	4.0	3.3	4.2	0.4	0.4	0.5	0.4
atinine	8.7	10.2	11.8	10.2	751	1019	1015	928	88	113	95	99

The calculation of «asparagine» is performed on the basis of an arbitrary I_c of 1.00 and a «mean amino acid ecular weight» of 130.

Table V. *Results in pregnant women (4, 5, 6).*

Amino acid	Plasma µg/ml				Urinary excretion µg/min.				Renal clearance ml/min. per 1.73 m²			
	4	5	6	Avg.	4	5	6	Avg.	4	5	6	Av
Taurine	5.7	5.2	4.3	5.1	—	—	6.1	—	—	—	1.4	—
Aspartic acid	0.9	1.1	1.0	1.0	0.7	1.4	0.9	1.0	0.8	1.4	0.9	1
Threonine	18.7	15.2	20.7	18.2	41.2	20.4	38.2	33.3	2.2	1.4	1.8	1
Serine	10.9	7.9	6.4	8.4	53.3	31.1	32.1	38.8	4.9	4.3	5.0	4
«Asparagine»*)	27.0	20.3	14.0	20.4	64.6	37.6	27.1	43.1	2.4	2.0	1.9	2
Proline	24.5	12.4	11.7	16.2	3.3	1.1	1.1	1.8	0.1	0.1	0.1	0
Glutamic acid	4.9	4.1	4.3	4.4	6.0	3.2	5.1	4.8	1.2	0.8	1.2	1
Glycine	14.0	8.1	6.5	9.5	225.5	98.6	133.8	152.6	16.2	13.2	20.4	16
Alanine	21.3	23.4	21.2	22.0	11.5	10.6	17.2	13.1	0.5	0.5	0.8	0
α-NH_2-butyric acid	1.4	1.6	1.1	1.4	4.0	6.1	3.4	4.5	2.9	4.1	3.1	3
Methionine	2.9	2.3	2.7	2.6	7.8	4.6	6.5	6.3	2.7	2.2	2.4	2
Isoleucine	6.1	7.5	5.5	6.4	8.4	7.0	6.2	7.2	1.4	1.0	1.1	1
Leucine	11.1	13.1	10.8	11.7	7.6	6.0	5.1	6.2	0.7	0.5	0.5	0
Tyrosine	4.0	5.3	4.9	4.7	8.6	13.1	16.7	12.8	2.2	2.7	3.4	2
Phenylalanine	6.0	6.4	6.1	6.2	4.7	4.9	8.8	6.1	0.8	0.8	1.4	1
Lysine	16.9	20.1	22.8	19.9	27.5	16.2	32.6	25.4	1.6	0.9	1.4	1
Histidine	10.3	9.2	11.5	10.3	149.4	156.0	179.3	161.6	14.6	18.3	15.5	16
1-CH_3-histidine	0.8	0.4	—	0.6	8.2	8.3	7.1	7.9	10.3	22.4	—	16
Arginine	9.8	6.7	6.3	7.6	5.4	3.9	4.5	4.6	0.6	0.6	0.7	0
Creatinine	6.8	8.4	10.0	8.4	826	1000	795	874	122	129	79	1

*) The calculation of «asparagine» is performed on the basis of an arbitrary Ic of 1.00 and a «mean am acid molecular weight» of 130.

of statistical analysis of the difference between the average clearances in the two groups are rendered. It appears that the clearances for α-aminobutyric acid, arginine, "asparagine", glutamic acid, glycine, histidine, leucine, lysine, methionine, serine, threonine and tyrosine are significantly higher for pregnant than for non-pregnant women. The clearances for isoleucine, proline, phenylalanine, alanine and aspartic acid are unchanged. The ratio between the mean values of the plasma amino acid concentration in pregnancy and in non-pregnant state is less than 1 for practically all the amino acids studied. Significantly lower average values are found for aspartic acid, glycine, phenylalanine, serine and tyrosine. T average value of the sum of plasma ami acids for which clearance determinations ha been carried out (Table VI), is found to significantly lower in the pregnant gro than in the non-pregnant group.

DISCUSSION

The results reported supplement the given previously as an elevated excreti of glycine, α-aminobutyric acid, "asparagin and glutamic acid was found during pr nancy. At the period in pregnancy invest ated, it has not been possible to confi increased excretion of phenylalanine (Wa

able VI. *Average values of clearances and of plasma amino acids for pregnant compared to non-pregnant women.*

Amino acid	Ratio between avg. values of clearances preg./non-preg.	Difference in clearances Preg. compared to non-preg. t*)	P†)	Ratio of plasma amino acid concentrations preg./non-preg.	Differences in plasma amino acid concentrations t	P
ɔleucine	0.85	0.59	0.6	0.86	1.48	0.2
oline	1.00	0	1.0	0.81	0.93	0.4
ıenylalanine	1.20	0.71	0.5	0.78	11.35	0.001
anine	1.20	0.71	0.5	1.05	0.64	0.6
ginine	1.47	4.21	0.01	0.73	1.58	0.2
partic acid	1.54	1.90	0.1	0.73	3.50	0.02
ucine	1.90	4.02	0.02	0.88	1.07	0.3
utamic acid	2.14	3.41	0.03	0.86	1.05	0.4
ethionine	2.15	6.88	0.01	0.77	2.64	0.06
ıreonine	2.17	3.62	0.02	1.16	1.09	0.3
rine	2.18	5.98	0.01	0.54	4.29	0.01
sparagine»	2.33	4.76	0.01	0.73	1.45	0.2
rosine	2.52	4.42	0.01	0.61	4.96	0.01
NH_2-butyric acid ...	3.06	4.97	0.01	0.53	2.06	0.1
stidine	3.45	10.21	0.001	0.96	0.33	0.8
ycine	3.66	5.46	0.01	0.30	3.20	0.03
sine	4.33	4.32	0.01	0.98	0.20	0.8
eatinine	1.11	0.65	0.6	0.82	1.42	0.2

t is found by the formula: t = difference of mean values $x\sqrt{n_1 n_2/(n_1+n_2)}$ / S.D.
P is the probability of identity derived from t and the degree of freedom (n_1+n_2-2).

ff, Brodie & Borden, 1952) and of iso-ıcine (Miller, Ruttinger & Macy, 1954). Haugen & Blegen (1953) have, under ysiological conditions found a very con-ınt ratio: true endogenous creatinine clear-ce/inulin clearance averaging 1.01 ± 0.07. our subjects no difference was found be-een the endogenous creatinine clearances the two groups. This fact is somewhat rprising, since Bonsnes & Lange (1950), cht (1951) and Page, Glendering, Dig-n & Harper (1954) have found an in-ased inulin clearance during the first 8 nths of pregnancy. The last-mentioned ɔup of authors found, that the enhanced tidine excretion during pregnancy is due an increased glomerular filtration, a de-ased reabsorption by the renal tubules and to a decreased rate of disappearance of histidine from the blood.

The experiments reported in this paper do not indicate an increased glomerular filtration of the plasma amino acids, and these compounds were not found to be enhanced in plasma. On the contrary, the concentrations of the various amino acids in plasma were generally decreased during pregnancy, some of them significantly. The increased clearances for several of the plasma amino acids must therefore be due either to a diminished tubular reabsorption or to tubular secretion. The last-mentioned possibility can of course not be excluded, cf. Kamin & Handler (1951).

It is known that normal pregnancy is usually accompanied by a decreased concen-

tration of the total non-protein nitrogen in the blood. Olsen (1932) ascribes this completely to a fall in the concentration of urea. Cadden & Faris (1936) find during the first six months of pregnancy a decrease both in urea nitrogen and in the rest of the non-protein nitrogen. This is confirmed by Bonsnes (1947) and Clemetson & Churchman (1955) who have determined the amino nitrogen levels during pregnancy. Steenstrup (1956) has recently found significantly lower values for uric acid in plasma during pregnancy. Our investigations seem to be the first on individual plasma amino acid levels in pregnancy and show that not all of the amino acids are lowered to the same degree. The non-uniform lowering of the concentration of the different amino acids contradicts a simple relationship between amino acid concentration and blood volume, the latter being increased during pregnancy (Bucht, 1951). It is likely that the diminution in the concentration of the amino acids in plasma is due in part to the excretion of larger amounts of amino acids with the urine.

Concerning the possible cause of the change in kidney function with regard to amino acids during pregnancy it may be relevant to mention that Brodie, Wallraff, Borden, Holbrook, Stephens, Hill, Kent & Kemmerer (1950) have reported aminoaciduria after ACTH and cortisone treatment, and that Bayliss, Browne, Round & Steinbeck (1955) have found increased adrenocortical activity during pregnancy.

SUMMARY

Ion exchange resin chromatography has been used for clearance determinations for a series of amino acids in 3 non-pregnant and 3 pregnant women (3rd—4th month). The clearances for α-aminobutyric acid, arginine, "asparagine", glutamic acid, glycine, histidine, leucine, lysine, methionine, serine, threonine and tyrosine were significantly increased during pregnancy. The concentration of plasma amino acids was generally found to be lower than in the non-pregnant state. The possible causes of the changes are discussed.

REFERENCES

Bayliss, R. I. S., Browne, J. C. McC., Round, B. J. & Steinbeck, A. W.: Plasma-17-hydroxycorticosteroids in pregnancy. Lancet *268 I,* 6 1955.

Block, R. J., Durrum, E. L. & Zweig, G. A manual of paper chromatography and paper electrophoresis. Academic Press Inc., New York, 1955.

Bonsnes, R. W.: The plasma amino acid and amino nitrogen concentration during normal pregnancy, labor, and early puerperium. J. Biol. Chem. *168,* 345, 1947.

— & Lange, W. A.: Inulin clearance during pregnancy. Fed. Proc. *9,* 154, 1950.

Borsook, H.: Micromethods for determination of ammonia, urea, total nitrogen, uric acid, creatinine (and creatine) and allantoin. J. Biol. Chem. *110,* 481, 1935.

Brodie,E. C., Wallraff, E. B., Borden, A. L. Holbrook, W. P., Stephens, C. A. L., jr., Hill, D. F., Kent, L. J. & Kemmerer, A. R.: Urinary excretion of certain amino acids during ACTH and Cortisone treatment of rheumatoid arthritis. Proc. Soc. Exper. Biol. Med. 285, 1950.

Bucht, H.: Studies on renal function in man. Scandinav. J. Clin. & Lab. Investigation. Suppl. *3,* 1951.

Cadden, J. F. & Faris, A. M.: The nonprotein urea, and rest nitrogen of the blood during normal pregnancy and the puerperium. Am. J. Obst. & Gynec. *32,* 421, 1936.

Clemetson, C. A. B. & Churchman, J.: Plasma amino-acid levels following protein ingestion by pregnant and non-pregnant subjects. J. Obstet. Gynaec. Brit. Emp. *62,* 390, 1955.

ze, A., Moore, S. & Bigwood, E. J.: On the desalting of solutions of amino acids by ion exchange. Anal. Chim. Acta *11,* 554, 1954.

ugen, H. N. & Blegen, E. M.: The true endogenous creatinine clearance. Scandinav. J. Clin. & Lab. Investigation *5,* 67, 1953.

nda, M.: Untersuchung des Harns gravider Frauen. J. Biochem. (Japan) *2,* 351, 1923.

min, H. & Handler, P.: Effect of infusion of single amino acids upon excretion of other amino acids. Am. J. Physiol. *164,* 654, 1951.

peller-Adler, R.: Über eine neue Methode zur quantitativen Histidinbestimmung und über deren Anwendbarkeit zur Untersuchung von biologischen Flüssigkeiten insbesondere von Gravidenharnen. Biochem. Ztschr. *264,* 131, 1933.

ller, S., Ruttinger, V. & Macy, I. G.: Urinary excretion of ten amino acids by women during the reproductive cycle. J. Biol. Chem. *209,* 795, 1954.

ore, S. & Stein, W. H.: Procedures for the chromatographic determination of amino acids on four per cent cross-linked sulfonated polystyrene resins. J. Biol. Chem. *211,* 893, 1954a.

— — A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. J. Biol. Chem. *211,* 907, 1954 b.

Lawrie, N. R.: The excretion of L(-)-tyrosine in urine. Biochem. J. *41,* 41, 1947.

Olsen, A.: Einige Untersuchungen über die Nierenfunktion bei Eklampsie und bei den damit verwandten Schwangerschaftserkrankungen. Thesis. Nyt Nordisk Forlag, København 1932.

Page, E. W., Glendering, M. B., Digman, W. & Harper, H. A.: The causes of histidinuria in normal pregnancy. Am. J. Obst. & Gynec. *68,* 110, 1954.

Sheft, B. B. & Oldham, H.: Amino acid intakes and excretions during pregnancy. J. Am. Dietet. Ass. *28,* 313, 1952.

Steenstrup, O. R.: Hypo-uricemia during pregnancy. Scandinav. J. Clin. & Lab. Investigation *8,* 263, 1956.

Stein, W. H.: A chromatographic investigation of the amino acid constituents of normal urine. J. Biol. Chem. *201,* 45, 1953.

Turba, F.: Chromatographische Methoden in der Protein-Chemie. Springer-Verlag, Berlin, 1954.

Voge, C.: A simple chemical test for pregnancy. Brit. Med. J. *2,* 829, 1929.

Wallraff, E. B., Brodie, E. C. & Borden, A. L.: Urinary excretion of amino acids in pregnancy. J. Clin. Investigation *29,* 1542, 1950.

Scandinav. J. Clin. & Lab. Investigation 62—66, 9, 1957.

ON URINARY EXCRETION OF MERCURY FOLLOWING ORAL ADMINISTRATION OF „ORADON" AND OTHER DIURETICS

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Publications on the excretion of mercury following oral administration of diuretics are scarce. When measuring Mercuhydrin according to radio-tracer technique, Overmann, Gordon & Burch (1950) obtained recoveries of only 5 per cent and Huffman (1949) found only up to 3 per cent in the urine after oral administration. Following oral administration of various mercurial diuretics Nuhfer & White (1951) found up to 6 per cent in the urine. CMPU (Chlormerodrin, Neohydrin) is recovered, *e. g.* by Moyer, Handley, Seibert & Snyder (1953), in quantities of 5—10 per cent in the urine. The intestinal absorption of the orally administered mercurial diuretics hitherto known thus seems to be very incomplete.

On parenteral administration (intravenous, intramuscular or subcutaneous) urinary recoveries of 60—100 per cent of the injected mercury are obtained (Vogl, 1953; Grossmann, Weston, Lehman, Halperin, Ullman & Leiter, 1951; Weston, Grossman, Lehman, Ullmann, Halperin & Leiter, 1951).

Oradon is a new mercurial diuretic which has proved to be particularly suitable for oral administration. It has been synthetized by A/S Ferrosan, and the chemical composition is: 3-hydroxymercuri-2-methoxy-succinimidopropane, theophylline, H_2O.

In the course of the first clinical obse vations of the diuretic effect obtained 30 patients (Hvidberg, 1956) the urina excretion of mercury was examined in patients. The results of these examinatio and the excretion of mercury following a ministration of other mercurial diuretics w be given in the following.

METHODS

According to Rolfe, Russel & Wilkinson (195 most of the methods for the determination of m cury suffer from the drawback that quantitat recovery of the mercury in the urine is not obtai if the urine has been allowed to stand for seve days. Since it has not been possible in the cou of the present investigation to start the analysis each of the collected samples of urine immediat on arrival, the method devised by Rolfe, Russel Wilkinson has been chosen.

Briefly, the method is as follows:

A quantity of urine corresponding to up to 250 mercury is oxidized for two hours in a glass pr sure bottle with nitric acid-permanganate at 85° Excess of permanganate is removed by means ammoniacal hydroxylamine solution, and the so tion is adjusted at pH = 8—9 by means of a monia. Sulphuric acid and hydroxylamine solu are added, and after suitable dilution the solut may be set aside until it is possible for the anal to continue the work — perhaps not until seve days have elapsed.

Table I. *Recovery of Hg.*

Sample	Hg in sample mcg./ml	Hg found mcg./ml	Percentage recovery
20.0 mcg./ml Oradon in water	6.84	7.0	102
		6.8	99.5
30.0 mcg./ml Oradon in water	10.2	10.1	99
		9.9	97
		12.5	100
30.0 mcg./ml Oradon in urine containing 2.3 mcg./ml Hg	12.5	12.7	101.5
		12.9	103
		12.7	101.5

Extraction is performed by means of dithizone toluene followed by reextraction by means of N hydrochloric acid. After addition of ammocal hydroxylamine the extraction is repeated, excess of dithizone is removed by means of ium hydroxide. The solution in toluene of the rcury-dithizone complex is measured in a Beckn spectrophotometer at 450 m μ. It is necessary run a blank.

The described method has proved to be very able. Recoveries obtained by the authors of the sent article agreed with the results given by fe, Russel & Wilkinson (Table I).

MATERIAL

The present investigation comprises, in addition the eight patients to whom Oradon was admiered, three patients to whom CMPU (in this the Danish preparation Asahydrin = Neohy-) was given orally and three to whom Thiomerin administered subcutaneously. As regards the don-patients the diuretic effect was examined, le with regard to the remaining subjects only urinary excretion of mercury was ascertained. ll 14 patients required diuretic therapy, two on unt of cirrhosis hepatis with ascites and edema, remaining on account of more or less marked gestive heart failure. None of the patients exaed suffered from any significant reduction of l function as measured by means of serum tinine. In the oral administration the preparawas given for four days, and the measurement the excretion of mercury was started on the before the administration began and continued until four days after the cessation of the treatment. Dosages appear from Table II. Earlier researchers have examined the urine for a period of up to 16 days after the cessation of the administration. No doubt traces of mercury would also be found at such a late date in some cases of Oradon administration, but after a period of four days the excretion is practically nil (see the curves). In the patients who were given Thiomerin subcutaneously the urine was examined from the day before to two days after the injection.

Urine was collected quantitatively, mixed and measured at 8 a.m. every day, and a sample of about 200 ml was withdrawn for the mercury analysis.

RESULTS

The results of the investigation appear from Table II and the curves of Figs. 1—4. For CMPU the excretion percentages found are slightly higher than those found by other authors, *viz.* 11—15 per cent. For Thiomerin recoveries are identical with those obtained by other authors, 66—97 per cent. Unlike other orally administered mercurial diuretics, Oradon is seen to result in a remarkably high urinary excretion of mercury, *viz.* on an average 54 per cent. The curves representing daily urinary excretion of mercury for all the eight patients agree fairly closely, and variations in percentage recoveries of mercury are comparatively small.

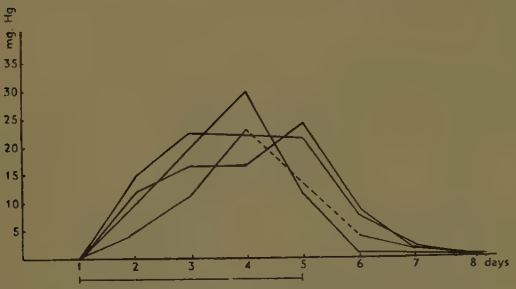

Fig 1 ORADON
DOSAGE 160 mg. Hg (40 mg per day)

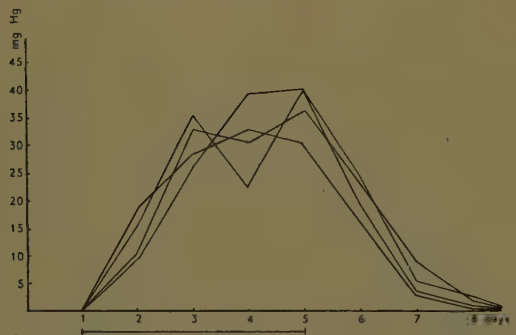

Fig. 2 ORADON
DOSAGE 240 mg. Hg (60 mg. per day)

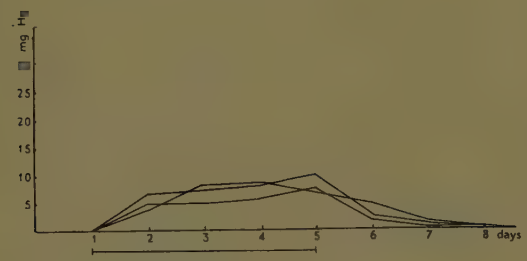

Fig 3 CMPU (ASAHYDRIN)
DOSAGE 240 mg Hg (60 mg per day)

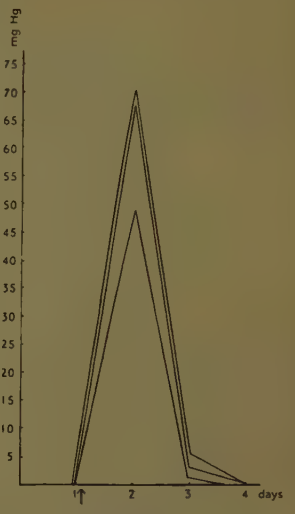

Fig 4 THIOMERIN
DOSAGE 80 mg subcutaneously

DISCUSSION

According to the present investigation the intestinal absorption of Oradon is more efficient than that of any previously examined mercurial diuretic given orally. This result has been obtained without any increase in the incidence of side effects (Hvidberg, 1956). The quantities of mercury caused pass through the kidneys by oral administr tion of Oradon are greater than those obtai able by treatment with other similar prepar tions.

It would be interesting if it were possib to ascertain a relationship between the high intestinal absorption of Oradon and the di retic efficacy as compared to the efficacy *e. g.*, CMPU. All other conditions bei equal, it might be expected that the high intestinal absorption would cause a certa dose of Oradon to produce the same diure effect as a greater dose of CMPU. On basis of the investigations hitherto made finite statements cannot, however, be ma as regards this question, but in Fig. 5 it been endeavoured to establish a comparis between two uniform materials. With rega to CMPU the figures are taken fr Grüner, Hilden & Krogsgaard's work

Table II.

Pat. No.	Drug	Dose in mg Hg daily	Dose in mg Hg altogether	Percentage recovery in urine	Average figures	
1	Oradon	40	160	49		
2	—	40	160	46	50	
3	—	40	160	*)		
4	—	40	160	57		54
5	—	60	240	61		
6	—	60	240	55	58	
7	—	60	240	56		
8	—	60	240	61		
9	Asahydrin	60	240	11		
10	—	60	240	14	13	
11	—	60	240	15		
12	Thiomerin	80	80	66		
13	—	80	80	97	83	
14	—	80	80	88		

*) Not less than 35 per cent. The excretion figure for the fourth day is lacking, for which reason the data for this patient have not been included in the average value.

amox (1955), while the Oradon figures e from Hvidberg (1956). The comparison ggests that the higher intestinal absorption Oradon is reflected in a higher diuretic ficacy. It should be emphasized, however, at the data are not absolutely commensur-le, and that the comparison cannot, there-re, form the basis of any exact evaluation the relative diuretic actions of the two ugs.

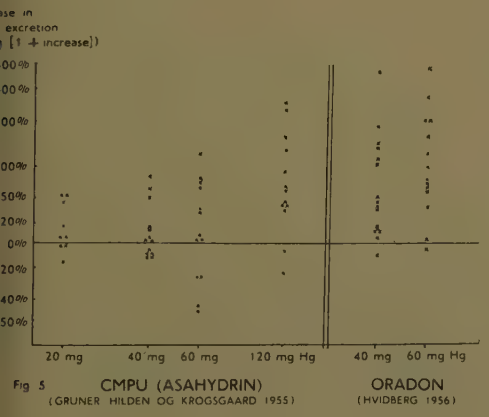

Fig 5

SUMMARY

Following diuretic therapy, quantitative determinations of mercury in urine from 14 patients have been made. Eight patients have been given a new mercurial diuretic, Oradon, which is administered orally, three patients have been given CMPU (chlormerodrin) orally and three patients have been given subcutaneous injections of Thiomerin (mercaptomerin sodium). Urinary recovery of mercury administered in the form of Oradon was on an average 54 per cent, while for CMPU the average recovery was 13 per cent.

REFERENCES

Grossman, J., Weston, R. E., Lehman, R. A., Halperin, J. P., Ullmann, T. D., & Leiter, L.: Urinary and fecal excretion of mercury in man following administration of mercurial diuretics. J. Clin. Investigation *30*, 1208, 1951.

Grüner, A., Hilden, T. & Krogsgaard, A. R.: Kliniske undersøgelser over den diuretiske virkning af acetazolamid (Diamox). Ugeskr. f. Læger *117*, 1541, 1955.

Hvidberg, E.: Oradon, et nyt kviksølvholdigt, peroralt diureticum. Ugeskr. f. Læger *118,* 1522, 1956.

Huffman, E. R.: Mercury excretion following oral mercurial diuretics in man. Am. J. Med. *6,* 663, 1949.

Moyer, J. H., Handley, C. A., Seibert, R. A. & Snyder, H. B.: Electrolyte, water and mercury excretion after oral administration of neohydrin. Arch. Int. Med. *92,* 847, 1953.

Nuhfer, P. A. & White, S.: A study concerning orally active mercurial diuretics. J. Pharm. Exper. Ther. *101,* 30, 1951.

Overman, W. J., Gordon, W. H. & Burch, G. E.: Tracer studies of urinary excretion of radi active mercury following oral administration a mercurial diuretic. Circulation *1,* 496, 195

Rolfe, A. C., Russell, F. R. & Wilkinson, N. T The absorptiometric determination of mercu in urine. Analyst, *80,* 523, 1955.

Vogl, A.: Diuretic therapy. Baltimore, Willian and Wilkins, 1953, p. 53.

Weston, R. E., Grossmann, J., Lehman, R. A., U mann, T. D., Halperin, J. P. & Leiter, L Renal extraction and excretion of mercury man following intravenously administered me curial diuretics. J. Clin. Investigation *30,* 122 1951.

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THE DISTRIBUTION OF CADMIUM IN BLOOD AFTER REPEATED EXPOSURE

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In chronic cadmium poisoning of man ild anemia has been observed (Friberg, 950). Severe anemic changes resulted from dmium poisoning in animal experiments Wilson, de Eds & Cox, 1941; Friberg, 950).

The cadmium content in the blood of rab-ts rose progressively in response to re-eated subcutaneous injections of cadmium lphate. After daily administration of 65 mg Cd per kg of body weight for about vo months concentration of cadmium in the ood was 1 to 2 μg per ml (Friberg, 1952), 955). It was shown that cadmium in the ood accumulated in the cells (Friberg, 952; Truhaut & Boudene, 1954). The tter writers, however, also found small uantities of cadmium in the plasma. The ntent of cadmium in the blood cells was 3 to 20 times that in the plasma.

We have further studied the distribution administered cadmium in the blood and ve attempted to illustrate the localization cadmium to the erythrocytes.

EXPERIMENTAL

Animal experiments: The cadmium used was belled with the radioactive isotope Cd^{115} * (half-e 43 days). At the beginning of the experiments the specific activity was approximately 1.5 mC per gram cadmium. Male albino rabbits were given subcutaneous injections of an 0.15 per cent aqueous solution of cadmium (0.65 to 1.00 mg Cd per kg of body weight) daily for one to six weeks. Blood was sampled two days to three weeks after the injections were discontinued. The specimens were taken from a vein of the ear or when the rabbits were killed by insertion of a cannula into the carotid artery.

Preparation of blood samples: A small quantity of heparin was added to the blood at the time of sampling. The plasma and cells were separated by centrifugation for 5 minutes at 2,000 r.p.m. The plasma was decanted and the cells were washed three times with physiologic saline. The erythrocytes were then hemolyzed by adding distilled water up to the initial volume of whole blood. The stroma was sedimented by centrifugation at 4000 r.p.m. for ten minutes. The clear supernatant fluid was pipetted off.

Dialysis: Continuous dialysis of about 20 ml of solution was carried out at + 4° C against a large volume of physiologic saline (about 300 ml) for 8 hours, with several changes of the diffusate. Polyvinyl tubes were used as dialyzing membrane.

Measurement of radioactivity: Radioactivity was measured on evaporated samples (approximately 80° C) in a Tracerlab windowless flow counter connected to a Tracerlab scaler. Comparisons were made with standard specimens. At least 600 impulses were counted in each test.

Electrophoresis: The hemolysate was examined with paper electrophoresis according to Kunkel & Tiselius (1951) and with zone electrophoresis in starch medium according to the column technique of Carlson (1954). Before electrophoresis the

* Supplied by the Atomic Energy Research tablishment, Harwell, England.

Table I: *Distribution of cadmium in the blood.*

Rabbit No.	Cadmium dose (mg/kg)	Exposure (weeks)	Examined after cessation of exposure (weeks)	Cadmium content							
				Whole blood		Plasma		Hemolysate before centrif.		Hemolysate after centrif	
				µg/ml	Per cent	µg/0.55 ml	Per cent	µg/ml	Per cent	µg/ml	Per cent
1	1.00	1	1/3	0.52	100	0.03	6	0.54	102	0.53	101
2	1.00	1	1/3	0.29	100	0.02	7	0.26	90	0.27	93
3	0.65	3	3	1.54	100	0.02	1	1.47	95	1.41	93
4	0.65	3	3	1.64	100	0.02	1	1.56	96	1.60	97

Table II: *Cadmium content of hemolysate and plasma before and after dialysis.*

Rabbit No.*)	Cadmium content				Dialyzable cadmium in per cent	
	Hemolysate after centrifugation (µg/ml)		Plasma (µg/0.55 ml)			
	before dialysis	after dialysis	before dialysis	after dialysis	Hemolysate	Plasma
1	0.53	0.30	0.03	0.03	43	ca 0
2	0.27	0.17	0.02	0.02	37	ca 0
3	1.41	0.82	0.02	—	42	—
4	1.60	1.07	0.02	—	33	—

*) Explanation see Table I.

sample was diluted with buffer. The amount of solution used for paper electrophoresis was 0.01 ml and for preparative column electrophoresis 1.0 ml. Veronal buffer was employed, pH. 8.6 and ionic strength 0.1 during 20 hours at + 4° C.

The paper electrophoresis strips were stained with bromphenol blue, after which they were washed with 0.5 per cent acetic acid. In column electrophoresis fractions of 1.3 ml were collected. From this volume 0.1 ml was taken for protein analysis with the modified Folin-Cicolteau method (Lowry, Rosebrough, Farr & Randall, 1951). The remainder was used for determining radioactivity.

RESULTS

The findings in cadmium analyses of whol blood, plasma, and hemolysate before an after centrifugation are presented in Table Almost all of the cadmium was found in th erythrocytes (hemolysate after centrifug tion). Only 1 to 7 per cent of the cadmiu content in whole blood was demonstrable the plasma. The concentration of cadmiu in the plasma was lowest when cadmium ha

Table III: *Cadmium content in hemolysate and in the hemoglobin peak after electrophoresis.*

Rabbit No.	Exposure (weeks)	Hemolysate µg/ml	Hemoglobin (electrophoresis) µg/ml	Hemoglobin (electrophoresis) Per cent recovered
5	2	1.02	1.09	107
6	2	0.82	0.74	90
5	4	2.53	2.46	97
6	4	2.10	2.06	98

een given for 3 weeks and the rabbits were illed after a further 3 injection-free weeks. s it was difficult to obtain plasma without race of hemolysis, however, the plasma alues are somewhat uncertain.

The results of dialysis are shown in Table I. After 48 hours dialysis against physiologic aline, about one-third of the erythrocyte ontent of cadmium was recovered in the ialysate. None of the plasma cadmium was emonstrably dialyzable. Because of the low admium concentration in the plasma, how-ver, the accuracy of the latter readings is oubtful.

Paper electrophoresis was carried out on ne samples taken after 1 to 3 weeks expo-ure to cadmium. No change in the mobility f the hemoglobin was observed and no new omponents appeared.

The zone electrophoresis diagram of the emolysate is shown in Fig. 1, in which the rotein concentration and the radioactivity the fractions are registered. This electro-horetic experiment was performed on a mple taken after 2 weeks exposure to cad-ium. The radioactivity followed closely the

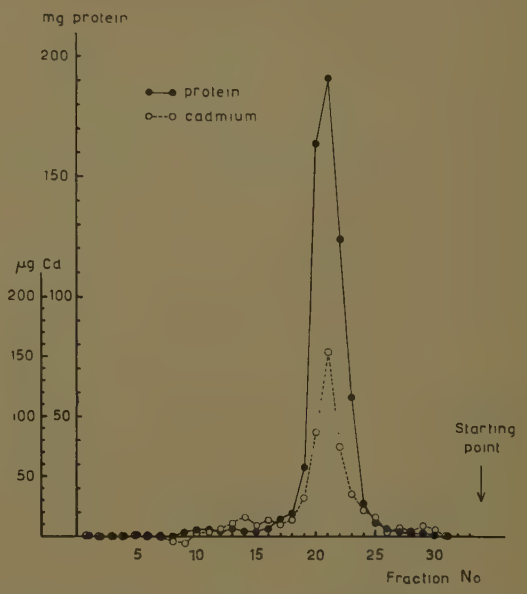

Fig. 1. Rabbit 1. Zone electrophoresis of hemolysate after 2 weeks exposure to cadmium. Anode to the left.

large protein component in the diagram.

The amount of cadmium recovered in the hemoglobin peak after electrophoretic separation is presented in Table III. As can be seen the recovery ranged from 90 to 107 per cent.

DISCUSSION

The analyses of whole blood, plasma and hemolysate showed that the erythrocytes contained more than 90 per cent of the cadmium in whole blood. The stroma did not appear to be the site of the metal cadmium.

Two-thirds of the cadmium was not dialyzable after dialysis against physiologic saline for 48 hours. Most of the cadmium, therefore, must have been bound to a macromolecule.

In zone electrophoresis the cadmium was quantitatively demonstrated in the hemo-

globin component of the diagram. This, together with the findings in dialysis, indicates that the cadmium in the blood after repeated injections was at least partly bound to the hemoglobin molecule. A highly probable association between other metals and hemoglobin has been reported. Thus Hunter, Kip & Irvine (1942) found that arsenic was concentrated in the erythrocytes, seemingly combined with the hemoglobin. Bahner (1954) demonstrated that antimony in the blood was localized to the erythrocytes and probably was bound to the globin fraction of hemoglobin. On the other hand, the findings of Watanabe & Yana (1952) indicated that in chronic lead poisoning of man, lead is mainly present in the erythrocyte stroma.

The anemia in animals and human subjects after exposure to cadmium may appropriately be stressed at this point. It seems possible that cadmium is transported to the bone marrow, where it inhibits the synthesis of hemoglobin and is partly incorporated in the hemoglobin molecule.

SUMMARY

The distribution of cadmium in the blood was studied in rabbits treated with subcutaneous injections of the isotope Cd^{115}. More than 90 per cent of the blood cadmium was found in the erythrocytes. Zone electrophoresis of hemolysate showed quantitative migration of cadmium with the hemoglobin.

REFERENCES

Bahner, C. T.: Localisation of antimony in bloo Proc. Soc. Exper. Biol. & Med. *86,* 371, 195

Carlson, L. A.: Electrophoretic studies of seru lipoproteins. I. A description of apparatus an technique for their separation in starch mediu Acta Chem. Scandinav., *8,* 510, 1954.

Friberg, L.: Health hazards in the manufacture c alkaline accumulators with special referenc to chronic cadmium poisoning. Acta Me Scandinav. *138,* suppl. 240, 1950.

Friberg, L.: Further investigations on chron cadmium poisoning. A. M. A. Arch. Ind. Hy and Occup. Med. *5,* 30, 1952.

Friberg, L.: Iron and liver administration i chronic cadmium poisoning and studies of th distribution and excretion of cadmium. Act pharmacol. et toxicol., *11,* 168, 1955.

Hunter, F. T., Kip, A. F. & Irvine, J. W.: Radio active tracer studies of arsenic injected a potassium arsenite. J. pharmacol. and expe therapeut. *76,* 207, 1942.

Kunkel, H. G. & Tiselius, A.: Electrophoresis c proteins on filter paper. J. Gen. Phys. *35,* 8 1951.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. Randall, R. J.: Protein measurement with th Folin phenol reagent. J. Biol. Chem. *193,* 26 1951.

Truhaut, R. & Boudene, C.: Recherches sur le so du cadmium dans l'organisme au cours d intoxications interet en medecine du trava Arhiv za Higijenu Rada *5,* 19, 1954.

Watanabe, G., & Yana, T.: Distribution of le in blood. III. The distribution of lead blood cells. Igaku to Seibutsugaku *22,* 2 1952, C. A. *46,* 10395, 1952.

candinav. J. Clin. & Lab. Investigation 71—79, 9, 1957.

SEPARATION AND IDENTIFICATION OF BARBITURATES N BIOLOGICAL MATERIAL BY PAPER CHROMATOGRAPHY

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In the clinical-chemical determination of rbiturates, ultraviolet spectrophotometric ethods are now generally used (Goldbaum, 948; Walker, Fisher & McHugh, 1948; ous, 1950, and others). The main clinical uestion is to ascertain the diagnosis *that* rbiturate is present in the material exained and only secondly to determine the *nount* present. In general the spectrophotoetric methods are fairly specific provided at determinations (on blood) are made in kaline and acid solution and at a number wavelengths and that "typical" barbirate absorption curves are obtained (see g. 3). In such cases spectrophotometric ocedures have also been used to differente between various barbiturates (Goldum, 1952). In some cases, however, the ectrophotometric methods are not satisctory. "Nontypical" absorption curves may obtained (see Fig. 5), when low amounts barbiturates are present and when interring substances are present, *e. g.*, after instion of a number of drugs other than rbiturates. Further, the spectrophotometric methods do not differentiate between active barbiturates and some metabolites with intact barbituric acid ring.

Paper chromatographic methods for the separation of common barbiturates have been described previously (Wickström & Salvesen, 1952; Algeri & Walker, 1952; Allgén, 1953). In order to check the spectrophotometric results in cases such as mentioned above paper chromatography was tried in this study.

METHODS

Spectrophotometric analysis was carried out in the first instance, followed by chromatographic analysis. The methods were tested with the following materials: urine, blood, gastric contents and tablets, though urine was the material mainly studied.

Paper chromatographic analysis.

For the general chromatographic technique reference is made to Lederer & Lederer (1953).

Extraction. 10 ml of blood (or more if available) was extracted once with 50 ml of chloroform by shaking for 3 minutes. After separation the chloroform phase was filtered and then evaporated to dryness on a water bath. The residue was triturated with 0.1 ml of ethanol for several minutes.

50 ml of urine, acidified by addition of a small amount of dilute sulfuric acid, was extracted with

Part of the analytical work was carried out the laboratory of Beckomberga Hospital, Brom-, Sweden. An abstract of this paper has been olished in Scandinav. J. Clin. & Lab. Investiga- 7 Suppl. 20, 124, 1955.

100 ml of chloroform. An aliquot (40 ml) was used for qualitative and semiquantitative spectrophotometric assay (after extraction into an alkaline aqueous phase) whereas the remainder was evaporated and the residue dissolved in 0.2 ml of ethanol. The same procedure was used with gastric contents. Tablet residues, if present, were collected by filtration through gauze and then extracted with chloroform.

Tablets were extracted with a small amount of ethanol or chloroform (1—2 ml/tablet).

Paper. Whatman No. 1 filter paper for chromatography was used in sheets 23 × 57 cm. With the buffered or sodium hydroxide containing solvents (II, III, IV, and V), the paper was immersed in the appropriate buffer or sodium hydroxide solution and was then air-dried for 24 hours.

Application of material. The final ethanol solutions from the various materials were applied as spots on the paper on a line about 7 cm from one end, and with a distance between spots of 2.5 or 3 cm. One and three times 5 µl of each solution were applied by micropipets. Standards were applied on each paper in amounts of 25—50 µg in ethanol solution. Diethyl-BA[1] is suitable as a general standard as its R_F value (in the alkaline solvents used) is intermediate between those of common barbiturates and barbiturate metabolites (Table I). Any barbiturate suspected in the specimen analysed was also applied as standard.

Apparatus. Generally descending chromatography was carried out using all-glass equipment.[2] The paper was fastened between two thin glass rods held together by small pieces of polyvinyl chloride tubing and was then easily handled (solvent I is irritating to the skin!) With solvent VII ascending chromatography was carried out (Allgén, 1953).

Solvents. Several solvents were tried, in general various alcohols (ethanol, butanol, *iso*amyl alcohol, tertiary amyl alcohol, hexanol, benzyl alcohol) co taining an alkaline aqueous phase (Wickströ & Salvesen, 1952; Algeri & Walker, 1952). N great differences were obtained between the systems, as regards separating power, as long an equal strength of alkali was used. The followi systems were studied in more detail or were us for special purposes:

I. *Iso*amyl alcohol-concentrated ammonium h droxide solution-ethylene glycol, 85 : 15 (v/v). The upper phase was used. *Isoam* alcohol and concentrated ammonium hydro ide were put in different containers on t bottom of the tank. Fresh ammonium h droxide was added before each run to ensu a saturated ammonia atmosphere.

II. *n*-butanol-0.1 *N* sodium hydroxide-ethyle glycol, 100 : 50 : 3 (v/v). The upper pha was used, the lower phase was put in a co tainer on the bottom of the tank.

III. same as II but pH 12 phosphate buffer us (250 ml 0.067 *M* Na_2HPO_4 and 250 0.067 *M* NaOH).

IV. same as II but pH 11.2 phosphate buff used (500 ml 0.067 *M* Na_2HPO_4 and 165 0.067 *M* NaOH).

V. same as II but 0.05 *M* pH 10 borate buff used.

VI. toluene-water-glacial acetic acid-ethylene gl col, 100 : 50 : 40 : 5 (w/w). The upper pha was used, the lower phase was put in a co tainer on the bottom of the tank (Wickstr & Salvesen, 1952).

VII. *n*-butanol-water-ethylene glycol, 100 : 50 (v/v).

Equilibration and running time. No equilibrati was found to be necessary with solvents I—V a VII, but with solvent VI the paper was allow to hang in the chromatography tank over ni before the solvent was filled in the through and t run thus started. With solvent I and VII t chromatogram was run for 20—24 hours. W solvents II—V for 15 hours and with solvent for 4 hours. With these running times the distan

[1] BA denotes barbituric acid.

[2] Supplied by the firm Werner-Glas, Stockholm, Sweden.

Table I. *R_F values of some barbituric acid derivatives in alkaline solvents.*

Substituent (R_2)	pK[1]	R_F value in solvent					$KMnO_4$ reaction
		I	II	II[1]	III	IV	
-dialkyl derivatives							
=ethyl							
thyl	7.91[2]	0.31	0.40	0.44	0.50	0.58	—
*so*amyl	7.94	0.61	0.69	0.72	0.73	0.79	—
-methylbutyl	8.11	0.61	0.65	0.74	0.75	0.78	—
-methyl-1-butenyl		0.45	0.51	0.56	0.58	0.63	+
henyl	7.41	0.34	0.40	0.47	0.48	0.54	—
*cyclo*hexenyl	7.50	0.45	0.53	0.55	0.57	0.66	(+)
*cyclo*heptenyl		0.50	0.57	0.58	0.63	0.67	(+)
=*iso*propyl							
-bromoallyl	7.72	0.43	0.53	0.61	0.62	0.69	+
=allyl							
*so*propyl	7.91	0.46	0.57	0.62	0.65	0.72	+
llyl	7.79	0.36	0.47	0.55	0.55	0.62	+
-butyl		0.58	0.65	0.70	0.74	0.74	+
henyl		0.39	0.44	0.50	0.53	0.57	+
methyl derivatives							
-methyl-5-*cyclo*hexenyl	8.34	0.58	0.60	0.64	0.69	0.73	(+)
,5-diethyl	8.45[3]	0.53	0.62	0.64	0.69	0.74	—
-ethyl-5-phenyl		0.53	0.59	0.61	0.65	0.67	—
-*iso*propyl-5(2-bromoallyl)		0.67	0.71	0.75	0.78	0.80	+
-dialkyl-2-thio derivatives							
-ethyl-5(1-methylbutyl)		0.68	0.74	0.74	0.76	0.78	(+)
-allyl-5(1-methylbutyl)		0.66	0.75	0.74	0.78	0.78	+
scellaneous derivatives							
arbituric acid	3.9[4]			0.02			—
-thio barbituric acid				0.04			(+)
-ethyl-5(3-hydroxy*iso*amyl) barbituric acid[5]		0.24	0.29	0.35	0.38	0.43	—

[1] R_F values in solvent II obtained at a lower mperature (18 ± 1° C) than the other determinations.

[2] $pK_1 = 7.85$, $pK_2 = 12.7$ spectrophotometrically ox & Shugar, 1952).

[3] value obtained spectrophotometrically (Fox & ugar, 1952).

[4] $pK_2 = 12.5$, both values obtained spectrophotometrically (Fox & Shugar, 1952).

[5] Synthesized according to Maynert (1952 a) using chromic acid oxidation.

pK1 values have been taken from Krahl (1940) unless otherwise stated. R_F values in general are means of five or more separate determinations. Permanganate reaction marked + is immediately positive, whereas (+) is a positive reaction obtained within 3 minutes.

travelled for the solvent front was about 35 cm. The temperature in the chromatography room in general kept within 21 ± 1° C.

Drying. After the run was completed the paper was allowed to air-dry (in a hood). With solvent VI drying was concluded in a drying oven, 30 minutes, at about 100° C in the presence of water vapor to remove acetic acid (which will decrease the sensitivity of the UV method of identification of the barbiturates).

Identification of spots

1) *Ultraviolet illumination.* The dry paper was inspected in a dark room with UV illumination.[1] Absorbing (and fluorescent) spots were marked in with a pencil. With chromatograms run in solvents I, VI and VII the paper was then treated with ammonia vapor in a closed container for a few minutes and then rapidly re-inspected with UV illumination and "new" spots marked in. The sensitivity of this identification method may be increased by the use of a fluorescent screen behind the paper (Grieg, 1952). In the present work only a faintly blue-white fluorescent sheet of filter paper, behind the paper inspected, was used.

To obtain a permanent reproduction of the chromatogram it may be copied on photographic paper with UV illumination (Fig. 1).

2) *Elution and ultraviolet spectrophotometry.* The spot was cut out from the chromatogram and extracted in a test tube with 4 ml of 0.05 *M* pH 10 borate buffer with occasional agitation for 30 minutes. A blank was obtained by treating similarly a non-absorbing, non-fluorescent part of the same chromatogram. The absorption curve for the solution in a 1 cm silica cell was then determined at various wavelengths between 220 and 350 mμ with a Beckman Model DU Spectrophotometer. There was then added 0.5 ml of 1 *N* sodium hydroxide solution to 3 ml of eluate and the absorption curve was redetermined ("pH 13"). Finally 1 ml of *N* sulfuric acid was added and the absorption curve redetermined ("pH 1"). With weak spots, smaller volumes and micro cuvettes were used.

[1] Analysis lamp with maximum emission at 2537 AU (supplied by Luma Ltd., Stockholm, Sweden. Type LUS 220, with filter).

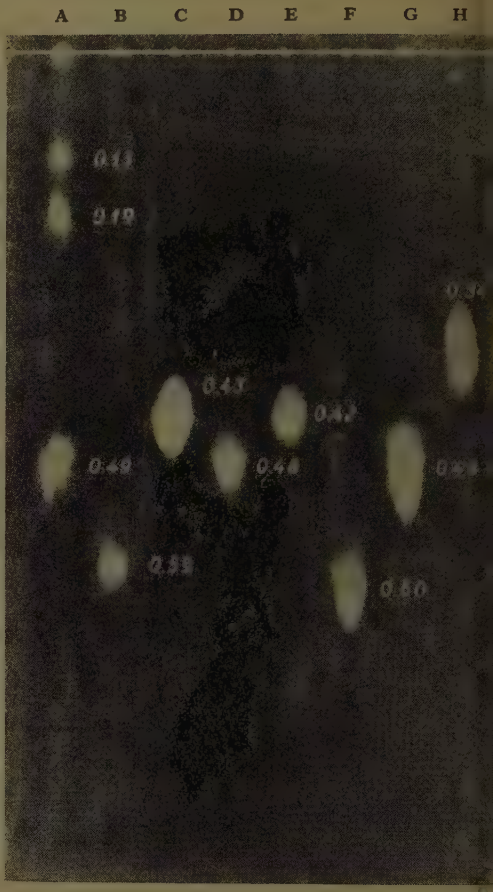

Fig. 1. Photographic reproduction of chromatogr in solvent I. Paper treated with ammonia vapor a illuminated for 15 sec with UV light. Distance fr lamp app. 40 cm. Gaevert document paper us Some of the spots are overloaded and show tailin With ethyl*cyclo*hexenyl-BA (A) two weak sp corresponding to oxidation products (R_F 0.13 a 0.19) are also shown. B = methyl*cyclo*hexenyl- methyl-BA, C = allylphenyl-BA, D = ethyl(1-m hylbutenyl)-BA, E = diallyl-BA, F = allyl-*n*-but BA, G = allyl*iso*propyl-BA, H = diethyl-BA. 5 150 μg was applied of each substance, R_F val are given at spots.

3) *Spraying reagents.* A number of chem identification methods were tried and the follow

ıich have been used by Wickström & Salvesen 952) were found most suitable and sensitive lgeri & Walker, 1952).

0.02 *N* potassium permanganate will give yellow r white) spots on a pink background with un-turated barbiturates within 3 minutes. With bar-urates containing a *cyclo*hexene or *cyclo*heptene ıg the colour develops more slowly than with saturation in an open chain (cf. Table I).

Spraying with 0.1 *N* silver nitrate — 10 per cent ımonium hydroxide (1 : 1. v/v) and drying for minutes at 95° C in a drying oven will give white ots on a brown background. This method was ed in some instances for the detection of diethyl-A and phenylethyl-BA after development with lvent VI.

RESULTS AND DISCUSSION

The R_F values obtained with a number of rbiturates in various alkaline solvents —V) are given in Table I. With increas-g alkalinity of the solvent better spread of F values is obtained and thus better separa-ons are achieved. The decrease of R_F values ith increasing pH is probably due mainly increasing ionization in the second ioniza-n step. Unfortunately the second ionization nstant of most barbiturates studied here is known so no comparison can be made be-een these constants and the R_F values tained. There is no obvious correlation tween pK_I and R_F values.

There is, however, some correlation be-een structure and R_F value. With N-ethylated barbiturates with only one ioniza-n step (Stuckey, 1940, Fox and Shugar, 52) higher R_F values are obtained than th the corresponding non-methylated bar-urates. Also there is a tendency towards gher R_F values with increasing length of open chain substituent.

In butanol-water (solvent VII) all com-on barbiturates give R_F values of the

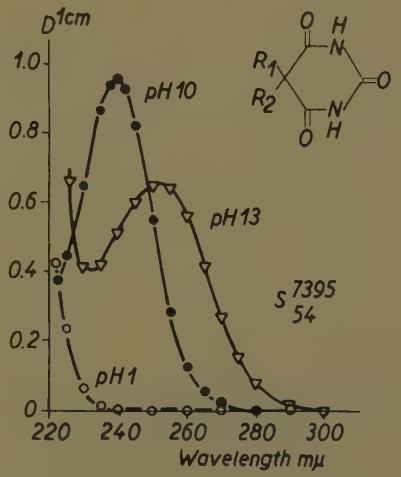

Fig. 2. UV absorption curves characteristic of 5.5-dialkyl barbiturates. Obtained by elution of spot corresponding to ethyl(3-hydroxy*iso*amyl)-BA on chromatogram in solvent I with urine from a patient who had ingested ethylisoamyl-BA. Elution with pH 10 borate buffer (•). Curves at pH 13 (Δ) after addition of alkali and pH 1 (o) after addition of acid are corrected to same concentration as at pH 10. (Stuckey, 1940; Lous, 1950; Fox & Shugar, 1952). D denotes absorbance.

order of 0.9. With oxidation products such as 5-ethyl-5(3-hydroxy*iso*amyl)-BA, slightly lower R_F values are obtained. With barbituric acid and thiobarbituric acid, R_F values close to zero have been obtained in this solvent as is also the case with alkaline solvents (Table I).

Solvent V was only used with part of the barbiturates listed in Table I. The R_F values found were intermediate between those found in solvent IV and VII. The separating power is low.

Among the alkaline solvents, *iso*amyl alcohol-ammonia (solvent I) was found to be the most suitable for the separation of barbiturates. Good separation between barbi-

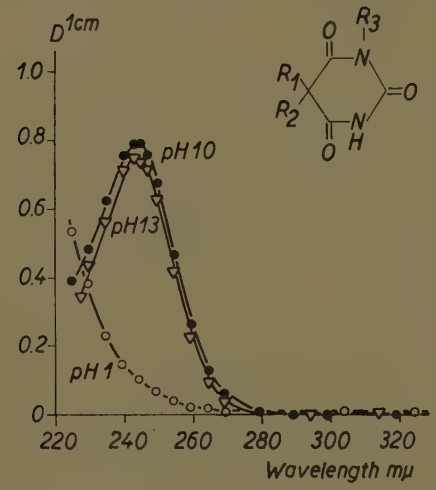

Fig. 3. UV absorption characteristic of N-alkyl barbiturates. — Obtained by elution of methyl-*cyclo*hexenyl-N-methyl-BA spot on chromatogram in solvent I. Notation etc. as in Fig. 2.

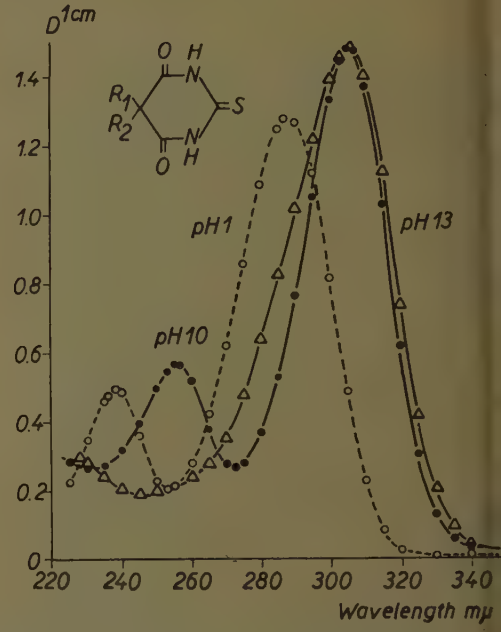

Fig. 4. UV absorption curves characteristic of 5,5-dialkyl-2-thio barbiturates. — Obtained by elution of allyl(1-methylbutyl)-2-thio-BA spot on chromatogram in solvent IV. Notation as in Fig. 2. (Brodie *et al.*, 1950).

turates is obtained in this solvent (R_F values between 0.3 and 0.7) and also between barbiturates and metabolites with intact BA ring but with additional polar groups such as —OH, >C=O and —COOH (R_F values between 0 and 0.3). Distinct spots are obtained with 20—25 μg barbiturate, whereas some tailing was obtained with higher amounts of barbiturate. These characteristics also apply to solvent II, which is easier to work with and has a less offensive odor. Solvent I is superior in one important respect, however: barbiturate spots are in general not identified by direct illumination of dry paper with UV light, but only after treatment with ammonia vapor. This is contrary to the case with a number of interfering substances. Thus the identification of barbiturates (and some metabolites) is more reliable after runs in solvent I than with the other alkaline solvents used.

Solvent I was also suitable for the sepa ration of barbiturate metabolites but bett separations may be obtained with less alkali solvents such as solvent IV. Some barbit rates have very close R_F values in solvent To obtain better separations in those case solvent VI was employed, *e. g.* to separa diethyl-BA and ethylphenyl-BA. With t amounts of barbiturates used, a tendency tailing was observed in this solvent.

By the determination of absorption curv after elution of spots, barbiturates may identified as such with high probability. general the determination of absorptio curves at two pH values (pH 10 and pH will be sufficient. Further, barbiturates ar

etabolites may be classed into different oups by this technique, *viz.* 5,5-dialkyl-BA ig. 2), 5,5-dialkyl-N-alkyl-BA (Fig. 3) d thio-barbiturates (Fig. 4). With some etabolites and oxidation products other pes of curves may be obtained.

An example of the use of the photographic entification technique is given in Fig. 1. ne absorbing spots appear as white areas. his method is more tedious and was less nsitive in my hands than UV inspection t may be used to reveal absorbing spots overed" by fluorescent substances.

The permanganate reaction was found eful to reveal unsaturated barbiturates nd metabolites). It worked well with un-ffered papers (solvent I, VI and VII). ne sensitivity was then about the same as ith UV inspection of alkaline papers, *i. e.*, out 10—20 μg barbiturate per sq.cm. For e with buffered papers the reagent may ve to be acidified with sulfuric acid. The action is not specific but will be obtained ith a number of substances (Lederer & ederer, 1953). Identification with the silver trate-ammonia reagent was less sensitive an UV inspection.

With the alkaline solvents, the R_F values ere dependent on temperature, as is shown Table I for solvent II. Higher R_F values e obtained with lower temperatures (Lede-r & Lederer, 1953). This source of error ay be eliminated by simultaneous runs with andards.

The strongly alkaline solvents may be ex-cted to cause some breakdown of barbi-rates. No evidence of such effects was served in the chromatographic studies, wever. (Cf. Fig. 4, where some break-wn probably took place during spectro-

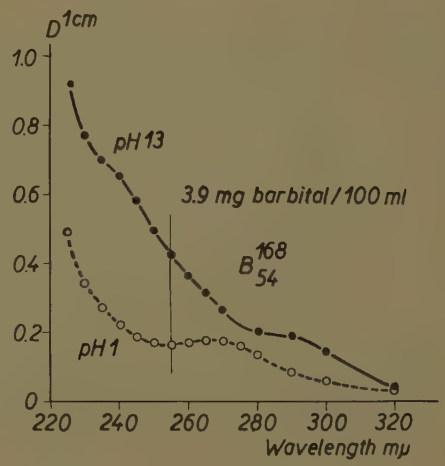

Fig. 5. Example of "non-typical" absorption curves. Spectrophotometric analysis of blood extract from a patient on the tenth day after ingestion of about 10 g of diethyl-BA. (Clinical diagnosis: barbiturate intoxication. On the first day typical barbiturate absorption curves were obtained showing a blood level of 19 mg/100 ml). Chromatography of blood showed dietyl-BA to be present.

photometry at pH 13, leading to a decrease of absorbance.)

In a few cases, with runs in solvents containing sodium hydroxide and with pH less than 11, two spots were obtained (with diethyl-BA and diallyl-BA) probably corresponding to monovalent and divalent salt. Similar results were obtained in trials with copper complexes where two spots, probably corresponding to uncombined and complex bound barbiturate, were obtained.

In Fig. 5 an example is given of "non-typical" absorption curves obtained on spectrophotometric analysis of blood extract. The curves do not justify a statement that barbiturate is present but, on chromatography one spot corresponding to diethyl-BA was ob-

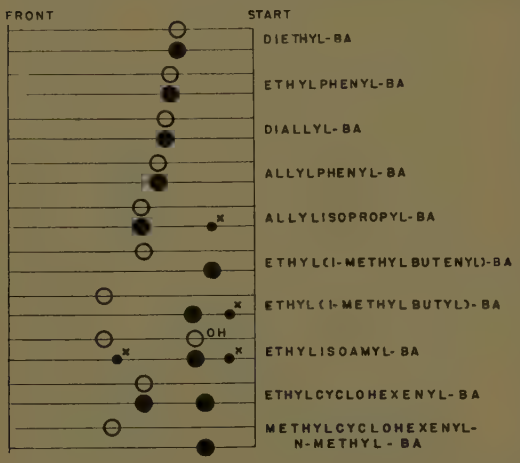

Fig. 6. Graphical representation of chromatographic results obtained with urines from a number of clinical cases having ingested various barbiturates. Open rings give position of standards, filled rings give position of absorbing spots from urine. — Solvent used: *iso*amyl alcohol-ammonia (I). The barbiturate nature of spots marked x has not been definitely established.

tained and this on elution showed typical barbiturate absorption.

Chromatographic findings for urines from a number of patients suffering from barbiturate intoxication, who had ingested various barbiturates, are surveyed in Fig. 6.[1] With the short acting barbiturates studied, UV absorbing metabolites appear in the urine extracts and are determined as barbiturates on spectrophotometric analysis. The spots given showed typical barbiturate absorption after elution except the spots marked x in the figure.

Although the metabolism of barbiturates is still incompletely known, a better understanding of its mechanism has been obtain in the last few years (Maynert & Van Dyk 1949; Maynert, 1952 a, b; Brodie, 195 Bush, Butler & Dickison, 1953; Algeri McBay, 1953, and others). It seems obvio that barbiturate metabolites must be kept mind as a source of error in the quantitati clinical-chemical determination of "activ barbiturates.

The chromatographic method does n seem suitable as a general routine meth for barbiturate analysis. The main disa vantage is the time taken for analysis, at lea 24 hours. The method is useful in speci cases, however: 1/ in forensic medici (Algeri & McBay, 1953), 2/ with deep unconscious patients to differentiate betwe long acting and short acting barbiturates a to exclude additional causes of unconsciou ness, 3/ when the diagnosis is uncertain, *e.g.* when "non-typical" absorption curves a obtained on spectrophotometric analys 4/ to increase certainty in the diagnosis a control of drug addiction, 5/ for separa determination of "active" barbiturates a UV absorbing metabolites. In most cases will be advantageous to investigate more th one of the materials: blood, urine, gast contents and tablets, if available. In this w information may be obtained both concerni unchanged barbiturate and metabolite(which will increase the certainty of the co clusion arrived at.

Because of the large number of barb turates in common use it will not be possib in all cases to elucidate the barbiturate p sent with the methods described here. Th applies especially to cases where very co

[1] These results will be reported in detail elsewhere.

x mixtures have been taken. The use of o-dimensional paper chromatography may necessary (*e. g.* solvent I and solvent VI).

ADDENDUM

Since this work was concluded Wright (1954) published results with a chromatographic tem using chloroform and buffered (alkaline) ers which seems more suitable as second solvent n the toluene-acetic acid solvent (VI) used here. ch systems have recently been developed further, ecially for medico-legal analysis (A. Frank, per- al communication).

REFERENCES

geri, E. J. & Mc Bay, A. J.: Paper chromatography of urinary metabolites of pentobarbital. Am. J. Clin. Pathol. *23,* 654, 1953.

& Walker, J. T.: Paper chromatography for identification of the common barbiturates. Am. J. Clin. Pathol. *22,* 37, 1952.

gén, L.-G.: Paper chromatographic identification of barbiturates. Analysis of urine in cases of acute poisoning. Svensk. Farm. Tidskr. *57,* 188, 1953.

odie, B. B.: Physiological disposition and chemical fate of thiobarbiturates in the body. Federation Proc. *11,* 632, 1952.

Mark, L. C., Papper, E. M., Bernstein, E. & Rovenstine, E. A.: The fate of thiopental in man and a method for its estimation in biological material. J. Pharmacol. & Exper. Therap. *98,* 58, 1950.

sh, M. T., Butler, T. C. & Dickison, H. L.: The metabolic fate of 5(1-cyclohexen-1-yl)-1,5-dimethyl barbituric acid (hexobarbital, evipal) and of 5(1-cyclohexen-1-yl)-5-methyl barbituric acid (nor-evipal). J. Pharmacol. Exper. Therap. *108,* 104, 1953.

s, J. J. & Shugar, D.: Absorption spectra and structure of barbituric acid derivatives as a function of pH. Bull soc. chim. Belges *61,* 44, 1952.

Goldbaum, L. R.: An ultraviolet spectrophotometric method for the determination of barbiturates. J. Pharmacol. & Exper. Therap. *94,* 68, 1948.

— Determination of barbiturates. Ultraviolet spectrophotometric method with differentiation of several barbiturates. Anal. Chem. *24,* 1604, 1952.

Grieg, A.: Paper chromatographic separation of barbituric acids. Nature *170,* 845, 1952.

Krahl, M. E.: The effect of variation in ionic strength and temperature on the apparent dissociation constants of thirty substituted barbituric acids. J. Phys. Chem. *44,* 449, 1940.

Lederer, E. & Lederer, M.: Chromatography. A review of principles and applications. Elsevier Publ. Co., Amsterdam 1953.

Lous, P.: Quantitative determination of barbiturates. A modified spectrophotometric method for clinical use. Acta Pharmacol. et Toxicol. *6, 227,* 1950.

Maynert, E. W.: Ethyl(3-hydroxy*iso*amyl) barbituric acid as the principal metabolite of amytal. J. Biol. Chem *195,* 397, 1952 (a).

— The distribution and fate of dialkyl barbiturates. Federation Proc. *11,* 625, 1952 (b).

— & Van Dyke, H. B.: The metabolism of barbiturates. Pharmacol. Rev. *1,* 217, 1949.

Stuckey, R. E.: The ultra-violet absorption spectra of barbituric acid and its 1-methyl and 1:3-dimethyl derivatives. Quart. J. Pharm. Pharmacol. *13,* 312, 1940.

Walker, J. T., Fisher, R. S. & Mc Hugh, J. J.: Quantitative estimation of barbiturates in blood by ultra-violet spectrophotometry. I. Analytical method. Am. J. Clin. Pathol. *18,* 451, 1948.

Wickström, A. & Salvesen, B.: The separation and identification of some barbiturates by paper partition chromatography. J. Pharm. Pharmacol. *4,* 98, 1952.

Wright, J. T.: A rapid method of identifying particular barbiturate derivatives in small samples of blood by paper chromatography. J. Clin. Pathol. *7,* 61, 1954.

Scandinav. J. Clin. & Lab. Investigation 80—84, 9, 1957.

KIDNEY FUNCTION STUDIES IN MYELOMATOSIS

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Few workers have attempted a functional approach to the problems of the myeloma kidney. Armstrong (1950) performed kidney function studies in 15 subjects suffering from myelomatosis, and Goldman, Adams & Luchsinger (1952) studied 9 cases.

Armstrong found a tendency towards better preservation of the tubular function than of the glomerular function and the renal plasma flow. Goldman *et al.* found a parallel reduction of the glomerular, tubular and vascular functions indicating destruction of entire nephron units. These results are in accordance with the widely accepted idea of the essential role of tubular obstruction in myelomatosis; a comparatively greater impairment of the glomerular function and the renal plasma flow than of the tubular function is more difficult to fit in with this view.

MATERIAL AND METHODS

Our material comprises 10 normotensive subjects suffering from myelomatosis, the diagnosis confirmed in all cases by bone marrow aspirations. Proteinuria was present without exception, but Bence-Jones protein was not found in cases 3, 4 and 8. Age and sex are recorded in Table I. Urethane was administered in cases 1 to 5 in the amounts recorded in the table and during the periods between the two dates stated. One subject (no. 4) had received urethane before the initial study, in all approximately 50 grams during t two preceding months.

We determined the glomerular filtration r (inulin clearance) and the renal plasma flow (pa aminohippuric acid clearance) in all cases — in of them both before and after treatment wi urethane. In 8 cases the tubular excretory maximu and in 7 cases the extraction ratio of para-amin hippuric acid were also determined. Methodolog ally we followed the descriptions of Goldring Chasis (1944), except in two cases (nos. 9 and 1 where the anthrone method for inulin determi tions (White & Samson, 1951) was used.

The extraction ratios were obtained by cath terisation of the right renal vein, blood samples spectrophotometric determination of the oxyg saturation (Klungsøyr & Støa, 1954) were tak at the same time.

All patients were studied in the post-absorpt state. Each value for glomerular filtration r renal plasma flow and tubular excretory maxim is the mean of three collection periods of cathe urine and is corrected to a body surface 1.73 sq. m. The values for extraction ratios also the average of at least 3 determinations.

RESULTS AND DISCUSSIONS

Our findings are presented in Table I

The frequency and progressive nature the kidney lesions in myelomatosis are f demonstrated by the results yielded by simpler tests — determination of blood u blood urea clearance and concentration pacity.

Case No.	Age (years) and sex	Time of study and urethane therapy	Blood urea (mg/100 ml)	Urea clearance: Max. in per cent of normal / Stand. in per cent of normal	Specific gravity (Addis & Shevky's test)	GFR (Cl_{IN}): Ml/min.	GFR (Cl_{IN}): In per cent of normal	RPF (Cl_{PAH}): Ml/min.	RPF (Cl_{PAH}): In per cent of normal	TM_{PAH}: Mg/min.	TM_{PAH}: In per cent of normal	Filtration fraction	Extraction ratio	«True» RPF: Ml/min.	«True» RPF: In per cent of normal	«True» filtration fraction	Oxygen saturation: Femoral artery	Oxygen saturation: Renal vein	Hemoglobin in per cent (100 per cent = = 14.8 g/100 ml)	Renal oxygen consumption (ml/min.)
1	65 M	Sept. 54	38	37	1022	37	29	153	24			0.24								
		100 grms.																		
		Nov. 54	77	20	1019	21	16	61	9			0.34								
2	67 M	Sept. 54	47	67		75	58	540	83			0.14								
		50 grms.																		
		Dec. 54				69	53	436	67			0.16								
3	63 M	Oct. 54	40	45	1018	94	72	544	84			0.17								
		100 grms.																		
		March 55	57	30	1022	75	58	524	81			0.14	0.84	568	87	0.13	97	89	77	10.2
						78	60			43	55									
4	68 F	Dec. 54	46	30	1022	79	66	305	51			0.26								
		50 grms.																		
		May 55	48	17	1019	60	50			50	65									
						69	58	322	54			0.21	0.75	391	65	0.18	97	82	82	14.7
5	55 M	Febr. 55	33	87	1027	97	75	531	82			0.18								
		150 grms.																		
		April 55	40	76		60	46			59	74									
						79	61	486	75			0.16	0.67	660	102	0.12	97	85	66	14.5
6	72 M	March 55	36	42	1013	43	33	277	43			0.15	0.73	345	53	0.13	94	82	80	10.1
						41	32			25	32									
7	51 M	June 55	36	88	1030	119	92	664	102			0.18	0.85	711	109	0.17	99	89	100	24.9
						127	98			70	90									
8	64 M	Jan. 56	73	31		48	37	177	27			0.27	0.80	201	31	0.24	91	79	68	4.6
						24	18			42	53									
9	61 M	June 56	104		1018	31	24	124	21			0.24								
						28	22			22	28									
10	61 M	Oct. 56	112	20	1012	13	10	43	6			0.29	0.19	231	36	0.05	98	84	66	5.8
						18	14			21	27									

When expressing the values for glomerular filtration rate, effective renal plasma flow and tubular excretory maximum as percentages of the normal mean, we have used the figures of Smith (1951) for normal males and females. On the whole the reduction of these functions is of approximately the same degree, and no special pattern of disturbed kidney function can be observed.

The average values for glomerular filtration rate, plasma flow and tubular excretory maximum are respectively 47 per cent, 51 per cent and 53 per cent of the normal in the eight cases in which all three functions were determined within a sufficiently limited period — a few days — as to warrant any comparison. The average value for glomerular filtration rate has been computed only from the determinations made simultaneously with registration of the renal plasma flow. The values for glomerular filtration rate registered while determining the tubular excretory maximum have been omitted, because a definite tendency towards a fall in the glomerular filtration rate was noted on administration of the more concentrated solution of para-aminohippuric acid.

Although we have found the tubular excretory maximum to be slightly less reduced than the plasma flow and the glomerular filtration rate, the difference is small, and our findings can scarcely be said to confirm the results reported by Armstrong. From his data it can be calculated that the average values for glomerular filtration rate, renal plasma flow and tubular excretory maximum were respectively 63 per cent, 66 per cent and 79 per cent of the normal. Our results are more in line with those of Goldman et al., whose corresponding figures can be calculated to be 73 per cent, 76 per cent an 69 per cent.

The values for filtration fraction tal reasonably well with both the observation of Armstrong and of Goldman et al., pro vided the calculation is based on the usu assumption of 100 per cent extraction o para-aminohippuric acid during the passag of the blood through the renal parenchym Armstrong points out the unavoidable erro involved in a calculation based on this a sumption in cases of definitely reduced ren blood flow, but as there appear to be n catheterisation studies in myelomatosis, in formation as to the extraction ration of th myeloma kidney has hitherto not bee available.

The values for the extraction ratios of ou cases — recorded in Table I — were, might be exected, definitely below the a cepted norm of 0.91, ranging from 0.19 0.85. The "true" values for renal plasm flow, obtained by multiplying by 0.91 t values for plasma flow computed from t renal arteriovenous difference in para-amin hippuric acid content $\left(\frac{Cl_{PAH} \times 0.91}{E_{PAH}}\right)$, a recorded in Table I, as are likewise t "true" filtration fractions. The differen between the ordinarily determined value f renal plasma flow and the "true" value marked in several cases; the average val for the "true" plasma flow is 69 per cent the normal.

Use of the factor 0.91 entails the assum tion that the proportion between the to renal plasma flow and the effective re plasma flow is the same in the diseased a in the normal kidney. It could well be, ho ever, that it is the absolute difference wh

mains approximately constant. If so, our lues for the "true" plasma flow are too gh. On recalculating these values, by as-ming a constant difference instead of a nstant proportion, only comparatively small anges are obtained in six of the eight cases. more marked change is apparent only in se 8 and case 10, and the average value r the "true" plasma flow is little changed. The low values for the "true" filtration action do not necessarily indicate an un-oportionally severe glomerular defect. The ssage of part of the blood through non-nctioning — "inert" — renal tissue might so provide a feasible explanation, as a struction of nephrons starting with tubular struction might in the beginning leave the omerular circulation relatively unaffected Smith, 1951). Both Armstrong and Gold-an et al. use the normal filtration fractions gistered in their materials as an argument ainst a primary glomerular defect. The sults of the catheterisation studies, how-er, indicate that caution should be exer-sed in the interpretation of the values for e plasma flow and the filtration fraction the myeloma kidney if these functions are termined without regard to the para-amino-ppuric acid content of the blood in the nal vein.

The results of the determinations of the ygen saturation in blood samples from the nal vein and the values for the simulta-ously determined arterial oxygen satura-n, are also recorded in Table I, and show at the renal arterio-venous oxygen dif-rence in myelomatosis is of the order dinarily encountered. In only one case was oxygen saturation below 80 per cent re-stered in the renal venous blood, but in this individual the arterial oxygen saturation was also subnormal. Without the values for oxy-gen capacity and hematocrit determinations, only a rough estimate of the renal oxygen consumption can be made on the basis of the values for hemoglobin content. The results (Table I), which are calculated from the values for the "true" renal blood flow, are in accordance with similar observations made in subjects with other kinds of renal lesions (Carghill & Hickam, 1949), a decreased oxygen consumption being observed in the more advanced cases.

The influence of the urethane treatment on the functional status of the myeloma kidney is probably negligible; in the 5 cases studied both before and after treatment a steady deterioration of the renal functions was registered. The doses administered were, however, relatively small and the intervals between the examinations somewhat pro-tracted.

SUMMARY

The values for renal plasma flow and glomerular filtration rate have been deter-mined in 10 cases of myelomatosis with pro-teinuria. In 8 of the cases determination of maximal tubular excretory capacity was also carried out. On the whole the results ob-tained indicate a parallel reduction of the glomerular, tubular and vascular functions of the myeloma kidney. Results of renal venous catheterisation studies — performed in 7 of the cases — indicate, however, that a relatively better preservation of the renal blood flow may exist. The renal arterio-venous oxygen difference was normal and the oxygen consumption varied with the degree of renal impairment.

REFERENCES

Armstrong, J. B.: A study of renal function in patients with multiple myeloma. Am. J. Med. Sc. *219,* 488, 1950.

Carghill, W. H. & Hickman, J. B.: The oxygen consumption of the normal and the diseased human kidney. J. Clin. Investigation *28,* 526,1949.

Goldman, R., Adams, W. S. & Luchsinger, E. B.: Renal function in multiple myeloma. J. Lab. & Clin. Med. *40,* 519, 1952.

Goldring, W. & Chasis, H.: Hypertension a hypertensive disease. The Commonwealth Fu New York, 1944.

Klungsøyr, P. & Støa, K. F.: Spectrophotometr determination of hemoglobin oxygen satur tion. Scandinav. J. Clin. & Lab. Investigati *6,* 270, 1954.

Smith, H. W.: The kidney. Oxford Universi Press, New York, 1951.

White, R. & Samson, F. E.: Determination inulin in plasma and urine by use of anthro J. Lab. & Clin. Med. *43,* 475, 1954.

candinav. J. Clin. & Lab. Investigation 85—88, 9, 1957.

NFLUENCE OF HEMOLYSIS AND TEMPERATURE ON THE PECTROPHOTOMETRIC DETERMINATION OF HEMOGLOBIN OXYGEN SATURATION IN HEMOLYZED WHOLE BLOOD

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During spectrophotometric determination the hemoglobin oxygen saturation in hemozed whole blood, the optical densities at 76 and 560 mμ were found to change with me after hemolysis with saponin and to epend on the temperature of the hemolyzed ood (Refsum & Sveinsson, 1956). Some vestigations on these phenomena are reorted in the present paper.

METHODS

All investigations were carried out on heparined, venous blood from healthy non-smokers in post-absorbtive, non-lipemic state. In order to oid the influence of a possible reactivation of me inactive hemoglobin during the investigations, samples were kept for at least 2 hours at room mperature before starting the experiments. All assware was thoroughly cleaned and sterilized.

For oxygenation, 5—8 ml blood was rotated for —20 minutes in a 300 ml tonometer, which was ushed with air or oxygen at intervals.

For hemolysis, 0.1 ml of a 30 per cent saponin lution (Merck) was added per 5 ml blood. The ponin solution was heated to boiling point and tered just before use. (Thus prepared and stored a refrigerator, this solution can be used for utine analyses for months.)

The optical densities were read on a Beckman ectrophotometer Model DU, using the ultrarocuvette described by Klungsøyr & Støa 954). The spectrophotometer was fitted with Dual Thermospacer Set and a thermostat circulan pump, allowing temperature control in the cell compartment and in the cuvettes. For further details see Klungsøyr & Støa (1954) and Refsum & Sveinsson (1956).

Optical density was read at 576 and 560 mμ, a maximum and a minimum in the absorption curve of oxyhemoglobin, and at 506 mμ, an isobestic point of the absorption curves of hemoglobin and oxyhemoglobin. While the *absolute* values read depend both on the depth of the cuvettes and on the concentration of total pigment, the *ratios* between the optical densities (D576/D560, D576/D506 and D560/D506) are dependent on the oxygen saturation only. For calculation of the hemoglobin oxygen saturation see Drabkin (1950).

RESULTS AND DISCUSSION

Changes with time after hemolysis.

After equilibration with air, the blood was drawn into two Luer syringes containing some glass beads. All air bubbles were carefully expelled and the syringes were sealed airtight. The blood in *one syringe* was hemolyzed at once and the optical densities read immediately and thereafter every 20th minute while the temperature was kept constant.

It was found that while D506 remained constant, D576 decreased and D560 increased until practically constant values were reached after 40—100 minutes. The changes of the ratios D576/D560, D576/D506 and D560/D506 in the same sample of blood, expressed in terms of decrease in hemoglobin oxygen

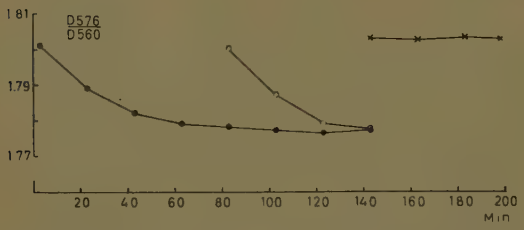

Fig. 1. Example of the changes of D576/D560 in whole blood with time after hemolysis with saponin. The total change of D576/D560 corresponds to a decrease in oxygen saturation of 1.8 per cent. Experimental temperature: 21° C.

●——●: After hemolysis immediately after equilibration with air.

o——o: After hemolysis 80 min. after equilibration with air.

x——x: After new equilibration of the hemolyzed blood with air.

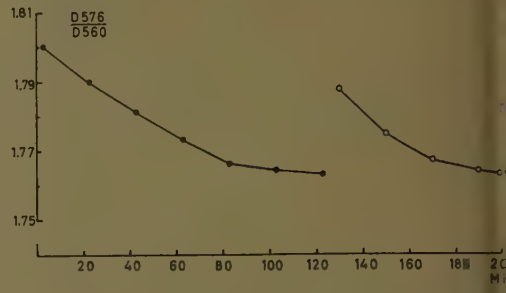

Fig. 2. Example of the influence of temperature o changes in D576/D560 in aerated whole blood wit time after hemolysis with saponin. The total chang of D576/D560 corresponds to a decrease in oxyge saturation of 2.7 per cent. Some of the hemolyze blood was examined at once at 22° C, while th remainder was kept at 7° C until the changes i the first portion were complete, and was then equi librated to 22° C and examined in the same manne

●——●: Reading immediately after hemolysis a 22° C.

o——o: Reading after standing at 7° C and the equilibration to 22° C.

saturation, were found to be practically identical. In blood samples from different donors the total decrease in oxygen saturation varied between 1.7 and 4 per cent.

The aerated blood sample in *the other syringe* was kept for 1—3 hours at room temperature before it was hemolyzed and the optical densities read as described above. The changes found in the ratios between the optical densities in this sample of blood were very nearly the same as those found in the sample hemolyzed immediately after equilibration.

When the optical densities in the hemolyzed blood reached constant values, the rest of the hemolyzed blood was again equilibrated with air and the optical densities read in the same way. Now the ratios between the optical densities remained constant, practically identical with the first values obtained immediately after hemolysis.

An example of the above observations is shown in Fig. 1. The same observations were done whether the blood samples wer kept for 2 or 8 hours before starting th experiments, and whether hemolysis wa produced by the usual or threefold quantit of saponin.

The rate of the changes of the optical den sities after hemolysis was also investigate at temperatures between 7° C and 37° C taking all readings at room temperature, an it was found to be considerably slower at lo temperatures. An example of the effect temperature is presented in Fig. 2.

These observations show that the change in optical densities observed in the bloo after hemolysis with saponin are not due t alterations of the hemoglobin itself, but t a decrease in the hemoglobin oxygen satura tion. Furthermore they indicate that th decrease is caused by a temporary increa

an oxygen-consuming process in the blood. his explanation is supported by the fact that e pH of the hemolyzed blood remained actically constant during the first hours er hemolysis (changing only a few hun-edths), and is in accordance with the obser-tions of Ramsey & Warren (1930).

In blood samples equilibrated with pure ygen instead of air before hemolysis, in-ring a surplus of physically dissolved ygen, no changes in the optical densities re observed.

Consequently, protracted spectrophoto-tric studies of oxyhemoglobin in hemo-ed whole blood, *e. g.* plotting of the ab-rbtion curve, should be carried out in blood turated with oxygen or in blood saturated th air *after* the cessation of the oxygen-nsuming process following hemolysis. In utine determinations of hemoglobin oxygen turation, the readings should be made *mediately* after hemolysis and furthermore a fixed temperature as emphasized below.

Influence of temperature

The optical densities of samples of fully ygenated, hemolyzed whole blood were ad at temperatures between 3° C and 37°C. was found that D576 decreased while 560 and D506 increased slightly when the nperature was raised, and *vice versa*. The tios between the optical densities varied shown in Fig. 3. The variations were re-rsible and practically the same in all mples, independent of whether the equili-ation was carried out with air or with ygen, or whether the equilibration, hemo-is and injection were performed at dif-ent temperatures. When calculated as anges in oxygen saturation per ±10° C, the

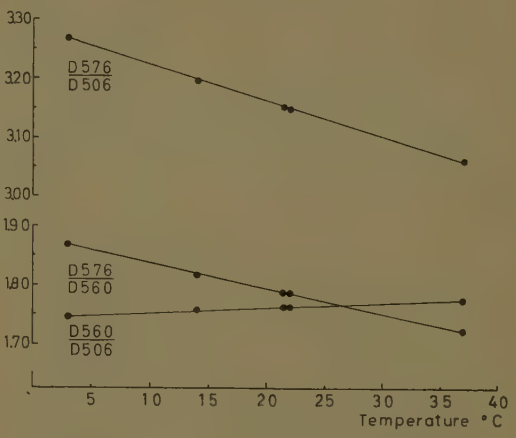

Fig. 3. Example of the influence of temperature on D576/D506, D576/D560 and D560/D506 in hemolyzed whole blood, equilibrated with air at 22° C. Readings were made first at 22° C, then at 3, 14, 22.5 and 37° C.

changes of the ratios D576/D560, D576/506 and D560/D506 corresponded to ∓ 2.7, ∓ 4.8 and ∓ 0.9 per cent respectively.

The variations of the optical densities could be ascribed neither to a drift of the maximum at 576 mμ or the minimum at 560 mμ towards other wave lengths, to temperature variation in the photocell, to changes of concentration due to temperature variations, nor to changes of the dissociation of the oxyhemoglobin.

In experiments both with blood with low hemoglobin oxygen saturation (8—10 per cent) and with blood reduced with sodium dithionite (6 mg/ml blood) after hemolysis, D576 increased while D560 and D506 decreased when the temperature was raised, and *vice versa*. Calculated as changes in hemoglobin oxygen saturation per ± 10° C the changes of the ratios D576/D560, D576/-D506 and D560/D506 in the blood reduced with sodium dithionite amounted to approxi-

mately ± 1.2, ± 2.6 and ∓ 2.3 per cent respectively.

As a result of the opposite changes of $DHbO_2 506$ and DHb506, the exact position of the isobestic point approaches 507 mμ when the temperature is raised, and 505 mμ when it is lowered.

From these experiments it appears that the influence of temperature on the optical densities of hemolyzed whole blood is dependent both on the wave lengths employed and the degree of oxygen saturation in the sample. Consequently accurate investigations should be performed at a fixed temperature. In determinations of oxygen saturation, the readings should be carried out at the temperature at which the constants in the calculation formula were determined. However, the linear relationship of the ratios between the optical densities and the temperature variations makes it possible to correct for temperature differences.

(For discussion of the influence of hemolysis and temperature on spectrophotometric determination of hemoglobin oxygen saturation in hemolyzed whole blood in another connection see Lambertsen, Bunce, Drabkin & Schmidt, 1952.)

SUMMARY

It is demonstrated that changes in the optical densities of newly hemolyzed whole blood kept from a gas contact in a closed system are due to a decrease in the hemoglobin oxygen saturation, probably caused by a temporary increase in an oxygen-consuming process in the blood. Protracted accurate studies of oxyhemoglobin in hemolyzed whole blood should be carried out in blood saturated with oxygen or with air *after* the cessation of the oxygen-consuming process.

It is demonstrated that the influence of temperature on the optical densities of hemolyzed whole blood is dependent both on the wave lengths employed and the degree of hemoglobin oxygen saturation. In accurate spectrophotometric investigations all readings should be performed at a fixed temperature.

REFERENCES

Drabkin, D. L.: Spectroscopy: Photometry and spectrophotometry. In: Medical Physics. Vol. II. The Year Book Publishers. Chicago 1950. p. 1076.

Klungsøyr, P. & Støa, K. F.: Spectrophotometric determination of hemoglobin oxygen saturation. Scandinav. J. Clin. Lab. Investigation *6*, 27, 1954.

Lambertsen, C. J., Bunce, P. L., Drabkin, D. L. & Schmidt, C. F.: Relationship of oxygen tension to hemoglobin oxygen saturation in the arterial blood of normal men. J. Appl. Physiol. *4*, 87, 1952.

Ramsey, R. & Warren, C. O., jr.: Rate of respiration in erythrocytes. Quart. J. Exper. Physiol. *20*, 213, 1930.

Refsum, H. E. & Sveinsson, S. L.: Spectrophotometric determination of hemoglobin oxygen saturation in hemolyzed whole blood. Scand. nav. J. Clin. Lab. Investigation *8*, 67, 1956.

dinav. J. Clin. & Lab. Investigation 89—94, 9, 1957.

CHANGES IN SERUM SULFHYDRYL AND SERUM GLYCOPROTEIN IN RHEUMATOID ARTHRITIS DURING TREATMENT WITH ADRENOCORTICAL STEROIDS

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An increase in serum glycoproteins, often considerable magnitude, has been found accompany a large number of clinical dis-ers of various entities. Elevated serum coprotein values have been observed in cer (Shetlar, Kelly, Foster, Shetlar & erett, 1950a), tuberculosis (Seibert, Sei-t, Atno & Campbell, 1947), diabetes erkman, Rifkin & Ross, 1953; Kei-g, 1954), rheumatoid arthritis (Shetlar, yne, Bullock, Patrick, Hellbaum & mael, 1953), (Shetlar, Payne, Padron, lton & Ishmael, 1956), rheumatic fever netlar, Schmidt, Lincoln, De Vore, Bullock Hellbaum, 1952) and in pregnancy (Shet-, Richmond & Everett, 1950b). The gin of this hyperpolysaccaridemia is not ar. The problems involved may, however, investigated from the point of view that perpolysaccaridemia is a result of a modi-d protein synthesis. In order to correlate e protein synthesis with the cause of the vated serum glycoprotein values, it would m to be desirable to detect the total amount serum sulfhydryl (serum-SH) present. ne reason for doing so is that cellular acti-y is partly based on the presence of ailable sulfhydryl groupes (Barron, 1953; mmet, 1934), and an alteration in -SH ll probably occasion a modified protein synthesis. The assumption that the -SH groups are involved during pathological processes which manifest themselves by increased serum glycoprotein is further supported by the fact that a reduced -SH serum content concurrently with a higher serum glycoprotein content has been shown in cancer by Stricks, Kolthoff, Bush & Kuroda (1953).

The present investigations indicate that in patients with rheumatoid arthritis there is an increase of serum glycoprotein and lowered serum -SH. Therapy conducted with adrenocorticoid steroids will tend to normalise the serum content of glycoprotein and, to a lesser extent, of -SH.

MATERIAL AND METHODS

The patients reported on in the present study, who were all suffering from clinically manifest rheumatoid arthritis, were admitted to the University Hospital of Copenhagen, Department A, for cortisone therapy. Blood samples were taken in the morning on fasting subjects. After clotting, the serum was kept at a temperature of + 2° C. Samples designed as normal were drawn from laboratory workers, nurses and patients with minor ailments. In the last-mentioned category the erythrocyte sedimentation rate, electrophoretic pattern and flocculation tests were all within normal limits.

Sulfhydryl groups were determined amperometrically according to the method of Kolthoff (1954). The buffer employed was n/10 phosphate,

pH 7.31, m/2 potassium choride. Titration was done with m/1000 mercuric acetate versus a standard calomel electrode at a voltage of —0.2 V. The indicator platinum electrode rotated with a speed of 1000 r.p.m. All titrations were carried out in nitrogen atmosphere, cleared of oxygen traces by passage through a column containing amalgamated zinc and 0.1 M. vanadyl sulphate, (Meites & Meites, 1948). In order to avoid foaming during nitrogen passage, 0.1 ml. Spand 85 (sorbitan trioleate, Atlas Powder Co.) was added to 1.0 l of the buffer solution. This addition does not interfere with titration results. A 1 ml microburette divided into two hundreths of a ml was used for all titrations. Results are expressed in Mmol -SH per 100 ml serum (1 Mmol -SH per 100 ml corresponds to 121 mg/100 ml cystein). Deviation on duplicate titrations did not exceed 3 per cent.

Serum polysaccarides were determined according to the anthrone method of Björnesjö (1955). This method, which eliminates the errors which have hitherto been observed on account of interference from tryptophane, yields an accuracy on each determination of 2 per cent. The results are expressed in mg of protein-bound non-hexosamine hexose in 100 ml serum.

Table I. *Protein-bound Hexose in Blo Serum of Normals and of Patients wi Rheumatoid Arthritis.*

Normals	mg/100 ml	Rheumatoid subject	mg/100 r
I	126	I a	173
II	118	II a	230
III	115	III a	177
IV	115	IV a	181
V	132	V a	165
VI	120	VI a	182
VII	122	VII a	118
VIII	128	VIII a	113
IX	132	IX a	187
X	124	X a	217
		XI a	230
$\bar{X}$:	123	XII a	171
S.D.:	6.0	XIII a	161
		XIV a	187
		XV a	204
		XVI a	174
		XVII a	195
		XVIII a	170
		XIX a	161
		XX a	165
		$\bar{X}$:	178
		S.D.:	29

RESULTS

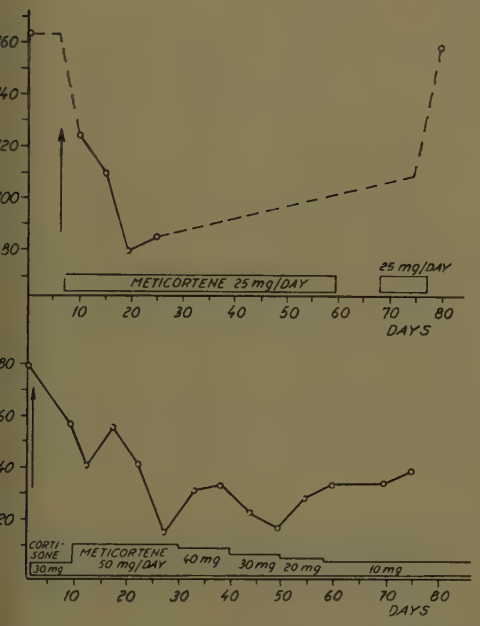
The anthrone method for determining serum glycoprotein on normal subjects showed a mean of 123 mg per 100 ml serum protein-bound non-hexosamine carbohydrate expressed as galactose-mannose in equal parts. The standard deviation on 10 determinations was 6.0 as shown in Table I. This is in agreement with the findings of Björnesjö (1955) who determined a mean of 123 mg per 100 ml with a standard deviation of 8.8 in 32 normal subjects. The titration of serum from 20 patients with rheumatoid arthritis resulted in a mean of 178 mg per 100 ml of protein-bound non-hexosamine carbohydrate. Although 18 of these 20 patients displayed values of more than 160 mg per 100 ml, the standard deviation was 29.

In Table II are shown the values f titrable -SH in serum from normal subjec and patients with rheumatoid arthritis. It seen that there is a clearly significa lowering of the -SH level in the group patients, the mean for which is 0.0390 Mm -SH per 100 ml of serum as compared wi 0.0620 Mmol -SH per 100 ml of serum the group of normal subjetcs. Goldbl (1954) determines the normal -SH in ser to 0.0552 Mmol per 100 ml. The standa deviation in the group of normals was 0.00 and in the group of patients 0.044.

Figure I shows the effect of therapy co ducted with adrenocortical steroids on t serum glycoproteins on two patients wi severe arthritis. As seen in the graph t

able II. *Content of Titrable Serum ulfhydryl Groups in Normals and Patients with Rheumatoid Arthritis.*

Normals		Rheumatoid subjects		
x Age	Mmol-SH per 100 ml serum	Sex	Age	Mmol-SH per 100 ml serum
36	0.050	f.	41	0.0425
45	0.050	f.	51	0.0405
42	0.049	f.	46	0.0375
33	0.080	f.	45	0.0317
35	0.064	f.	45	0.0380
42	0.063	m.	49	0.0410
37	0.059	f.	38	0.0365
51	0.061	f.	36	0.0495
28	0.064	f.	52	0.0380
36	0.063	m.	32	0.0405
$\bar{X}$ =	0.062		$\bar{X}$ =	0.0390
S.D.:	0.0077		S.D.:	0.0044



. 1. Effect of administration of cortisone and ticortene on the content of serum polysaccharides two subjects with rheumatoid arthritis. Onset of therapy indicated by arrow.

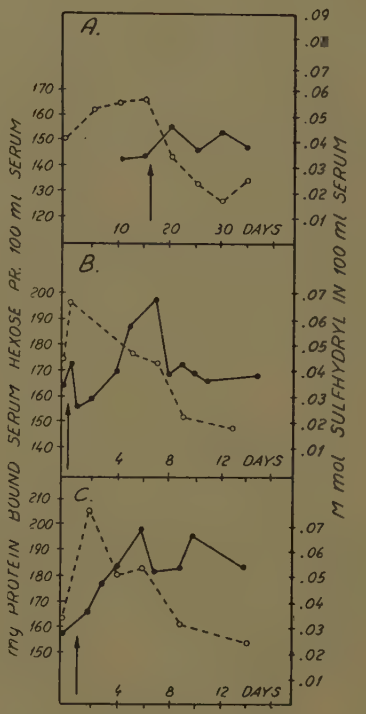


Fig. 2. Changes in serum sulfhydryl -●—●- and serum polysaccharide -o—o- during treatment with cortisone and meticortene of three subjects with rheumatoid arthritis. Dosages of steroids: A. 3 × 5 mg meticortene daily, B. 2 × 5 mg meticortene daily, C. 3 × 25 mg cortisone daily. Onset of therapy indicated by arrow.

serum glycoprotein values decrease during the course of therapy. The arrows indicate the onset of the therapy. In Figure 2, three patients have been investigated with regard to -SH and serum glycoprotein during a period of treatment with corticoids (cortisone and meticortene). Also in these instances the serum glycoproteins manifest a pronounced tendency to decrease, whereas serum -SH will increase during the same period. Sulfhydryl titrations in cases B and C Fig. 2 were

carried out not later than after two hours of storing in the ice box. In case A Fig. 2 the oldest of the first blood samples had been stored 21 days in the cold. This causes a delay of the labile -SH groups and therefore we determined the rate of sulfhydryl oxidation which was found to be 3 per cent per day. The -SH values in this case were corrected with this factor. These changes in serum -SH and serum glycoproteins were accompanied by considerable improvement in the clinical condition of the patients.

DISCUSSION

Important activities have been attributed to sulfhydryl compounds in recent years. The investigations carried out by Wentworth & Billows (1952) of synergistic action of cortisone on radiation injury, by Mazur, Litt & Shorr (1950) of vasotropic substances in shock and by Conn, Louis & Johnston (1949) of diabetes caused by cortisone or aloxan, leads to the conclusion that sulfhydryl groups may be involved in important metabolic processes. The present study investigates a correlation between -SH and serum glycoprotein. The reason for studying cases of rheumatoid arthritis is that in this condition an abnormal glycoprotein synthesis takes place, Table I. As will appear from Table II, a decrease in serum -SH is found also in this disorder. This leads one to believe that the reduced content of serum -SH might indicate a modified protein synthesis which should promote a greater tendency to glycoprotein formation.

The action of adrenocortical steroids on serum glycoproteins and serum -SH seen in Fig. 1 and 2 may be interpreted as an action of cortisone and meticortene on the glycoprotein synthesis, probably *via* an actio on serum -SH. *In vitro* experiments carrie out in this laboratory on the action of corti sone on serum showed that cortisone *in vitr* did not alter the serum -SH using cortison in therapeutic concentrations. Elevating th concentrations of cortisone about 1000 time the therapeutic concentration, an action wa seen which could be interpreted as an almos stoichimetrical reduction of serum -SS t -SH with cortisone -OH (Larsen, 1956). I has often been shown that cortisone impair protein synthesis. Clark (1953) pointed ou that one of the actions of cortisone is to limi protein synthesis, not by accelerating the pro tein catabolism but by inhibiting or regulatin the protein synthesis. A recent study b Lowe & Rand (1956) indicates an action o cortisone on the RNA metabolism. Histo chemically Teilum (1950) showed a simila action of cortisone and in 1951 Teilu demonstrated an inhibiting action of corti sone on the activity of plasma cells and th reticulo-endothelial system in hyperimmun zed rabbits. The assumption that modifie protein synthesis intensifies an abnorma glycoprotein synthesis may be evidenced fro the findings herein presented. Recent histo chemical findings of Teilum (1956) suppo the idea that a modified protein synthes may lead to an abnormal glycoprotein sy thesis.

SUMMARY

Serum polysaccharides were determin on 10 healthy subjects and 20 patients su fering from rheumatoid arthritis, and an i crease of from 123—178 mg/100 ml protei bound hexose was demonstrated. Seru sulfhydryl was determined on 10 healt

ubjects and 10 patients with rheumatoid rthritis and here a considerable decrease vas demonstrated, ranging from 0.062 Mmol SH per 100 ml serum in normals to 0.0390 Mmol sulfhydryl in patients.

During a period of therapy with adrenoortical steroids conducted on 5 patients, a ecrease in serum glycoprotein was demontrated. In the same period 3 of these 5 atients were examined for serum sulfhydryl nd the low pre-treatment values were heighened during the course of therapy.

Evidence for a closer relationship between hese findings has been discussed with special eference to the action of sulfhydryl groups n glycoprotein synthesis.

ACKNOWLEDGEMENT

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REFERENCES

Barron, E. G. S.: The importance of sulfhydryl groups in biology and medicine. Texas rep. Biol. M. *11,* 653, 1953.

Berkman, J., Rifkin, H. & Ross, G.: The serum polysaccharides in diabetic patients with and without degenerative vascular disease. J. Clin. Investigation *32,* 415, 1953.

Björnesjö, K. B.: Analyses of protein-bound serum polysaccharide with the anthrone reagent. Scandinav. J. Clin. & Lab. Investigation *7,* 147, 1955.

Clark, I.: The effect of cortisone upon protein synthesis. J. Biol. Chem. *200, 69,* 1953.

Conn, J. W., Louis, L. H. & Johnston, M. W.: Metabolism of uric acid, glutathione and nitrogen, and excretion of "11 oxysteroids" and 17 ketosteroids during induction of diabetes in man with pituitary adrenocorticotropic hormone. J. Lab. & Clin, Med. *34,* 255, 1949.

Goldblum, R. W., Piper, W. N. & Olsen, C. J.: The effects of ultraviolet light on the sulfhydryl and disulfide groups of normal human sera. J. Invest. Derm. *22,* 463, 1954.

Hammet, F. S.: Natural chemical factors in growth and development. Cold Spring Harbour Symposia Quant. Biol. *2,* 78, 1934.

Keiding, N. R.: Levels of serum protein fractions in diabetic patients with retinitis proliferans. Proc. Soc. Exper. Biol. Med. *86,* 390, 1924.

Kolthoff, I. M., Stricks, W. & Morren, L.: Amperometric titration of sulfhydryl groups in biologically important substances. Anal. Chem. *26,* 366, 1954.

Larsen, B.: Sulfhydryl groups in serum proteins during influence of cortisone. Paper read at the joint meeting of the Scandinavian Biochemical Societes in Oslo, June 4—5, 1956.

Lowe, C. U. & Rand, R. N.: Alterations in biochemical composition and ribonucleic acid metabolism induced in rat liver by cortisone. J. Biophysic. Biochem. Cytol. *2,* 331, 1955.

Mazur, A., Litt, I. & Shorr, E.: Relation of sulfhydryl groups in ferritin to its vasodepressive activity. J. Biol. Chem. *187,* 485, 1950.

Meites, L. & Meites T.: Removal of oxygen from gas streams. Anal. Chem. *20,* 984, 1948.

Seibert, F. B., Seibert, M. V., Atno, A. J. & Campbell, H. W.: Variation in protein and polysaccharide content of sera in the chronic diseases, tuberculosis, sarcoidosis and carcinoma. J. Clin. Investigation *26,* 90, 1947.

Shetlar, M. R., Kelly, K. H., Foster, J. V., Shetlar, C. L. & Everett, M. R.: Serum polysaccharide levels in pregnancy, parturitian and the post partum state. Am. J. Obst. & Gynec. *59,* 1140, 1950a.

— Payne, R. W. & Bullock, J. A., Patrick, D. R., Hellbaum, A. A. & Ishmael, W. K.: Comparative studies of serum polysaccharides in rheumatoid arthritis and degenerative joint disease. J. Clin. Investigation *32,* 1208, 1953 b.

— Payne, R.W., Padron, J., Felton, F. & Ishmael, W. K.: Objective evaluation of patients with rheumatic diseases. J. Lab. & Clin. Med. *48,* 194, 1956.

Shetlar, M. R., Richmond, V. & Everett, M. R.: Polysaccharide content of serum fractions in carcinoma, arthritis and infections. Cancer Research, *10,* 681, 1950b.

— Schmidt, H. L. Lincoln, R. B., De Vore, J. K., Bullock, J. A. & Hellbaum, A. A.: Response of serum polysaccharide fraction and protein fractions following cortisone treatment of patients with rheumatic fever. J. Lab. Clin. Med. *39, 372,* 1952.

Stricks, W., Kolthoff, I. M., Bush, D. G. & Kuroda, P. K.: Experimental study of the polarographich cancer test and of the sulfhydryl titration for the differentiation between normal, cancerous and other pathological sera. Lancet *73,* 328, 1953.

Teilum, G.: Periodic acid-schiff-positive reticuloendothelial cells producing glycoprotein: Functional Significance during formation of amyloid. Am. J. Pathol. *32,* 945, 1956.

— Engbæk, H. D., Harboe, N. & Simonsen, M.: Effects of cortisone on experimental glomerulonephritis. J. Clin. Pathol. *4,* 301, 1951.

— Engbæk, H. D. & Simonsen, M.: Effects of cortisone on plasma cells and reticulo-endothelial system in hyperimmunized rabbits. Acta Endocrinol. *5,* 181, 1950.

Wentworth, J. H. & Billows, J. A.: Synergistic Action of cortisone and total-body irradiation. Radiology, *59,* 559, 1952.

Technique and Practical Problems

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A SIMPLE APPARATUS FOR ULTRAFILTRATION

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In our studies of protein fractions from the preparative zone electrophoresis of human blood serum, it has been necessary to concentrate the dilute solutions under conditions which are as mild as possible in order to avoid denaturation. Ultrafiltration is a method which has been employed by several authors (Esser & Heinzler, 1952; Sober Gutter, Wyckoff & Peterson, 1956). In the following a simple ultrafiltration apparatus will be described which we have found to be simple and convenient for this and similar purposes.

Fig. 1. Support of plexi-glass for the ultrafilter.

PRINCIPLE

Over a suitably constructed cylindrical support of plexiglass (Fig. 1) is drawn a moistened dialysis membrane, approximately three times the length of the support. The membrane is sealed with the aid of rubber bands which — above — are wound tightly about the membrane and the rubber packing surrounding the evacuation tube and — below — about the end of the membrane which has been folded several times. The lower part of the membrane is folded up along the support and fastened with a rubber band after which the filter is evacuated to approximately 10 mm Hg. The filter is subsequently lowered into a graduated cylinder containing the solution to be concentrated.

Evacuation of the ultrafilter is performed with the aid of a vacuum pump or an effective water suction pump with a backflow valve which is connected to the filter *via* a reservoir flask for the filtrate, and a vacuum meter which also serves to indicate whether the membrane is intact after assembly (Fig. 2).

DISCUSSION

By means of the apparatus described above, an ultrafilter is attained which is simply, rapidly and inexpensively assembled. Its capacity naturally depends on the porosity of the membrane used. Here a membrane has been chosen (made by the Viking Corp., Chicago, U. S. A.) which makes the filter air-tight, even when evacuated to approximately 10 mm Hg. Thus, even when not fully submerged in solution, the filter nevertheless retains the vacuum and it is possible to concentrate to volumes of approximately 0.1 ml. For dilute solutions of low viscosity the capacity under these conditions is approximately 14 ml/hrdm^2, which, for example, corresponds to about 10 ml per hour for the filter shown in Fig. 1, which has a

Fig. 2. Set-up for ultrafiltration with 2 filters. The vacuum pump is connected *via* the tube at the right. Note: The valve is opened before the pump is stopped in order to prevent back suction.

diameter of 25 mm and a length of 90 mm. These dimensions, however, can be varied considerably. But it should be mentioned that the capacity is lowered with increased viscosity of the solution.

Another advantage of the air-tight membrane is that after the filter has been evacuated it is no longer necessary that the vacuum pump be connected to the system. Instead, the filter may be sealed and the pump disconnected after which ultrafiltration may be performed in a refrigerator, *etc* There is only an insignificant decrease i the vacuum and the vacuum is sufficient t cause the filling of the filter without furthe evacuation. Thus, using only one vacuum pump, as many filters as desired may b operated simultaneously. This is advanta geous when several different solutions o large volumes are to be concentrated, fo several different filters may be employe to enable a more rapid ultrafiltration.

Due to the principle of the filter, *viz.* wit the solution to be concentrated outside th membrane in a graduated cylinder, it is eas to arrange for the filtration to be automat ically discontinued at a predetermined volum or concentration, if the initial concentratio is known. This may be done simply by level ling the bottom of the filter with the desire volume mark on the graduated cylinder. Th filtration may thus proceed without furthe observation, so that the night may be utilized

SUMMARY

A simple apparatus for ultrafiltration an concentration of solutions of proteins an similar compounds of high molecular weigh has been described. The ultrafilter consist of a dialysis frame of plexi-glass, which i evacuated.

REFERENCES

Esser, H. & Heinzler, F.: Method of obtainin proteins for electrophoresis on filter pape from cerebrospinal fluid and other dilute pro tein solutions by filtration under pressure. Klin. Wchnschr. *30,* 600, 1952.

Sober, H., Gutter, F., Wyckoff, M. & Peterson, E. Chromatography of proteins. II. Fractionatio of serum protein on anion-exchange cellulos J. Am. Chem. Soc. *78,* 757, 1956.

andinav. J. Clin. & Lab. Investigation 97—98, 9, 1957.

LARGE SCALE SEPARATION OF PORPHYRIN PIGMENTS ON CELLULOSE POWDER COLUMNS

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(Received for publication August 25, 1956)

In a previous paper (Eriksen, 1953) a mple chromatographic method for the sepa- ıtion of porphyrin pigments on paper sheets ccording to the number of acidic groups of e pigments has been described. The method ould not, however, be used for the separa- on of larger amounts of porphyrins, and e have therefore tried to modify the method a column method. It was found that the orphyrins could be separated on cellulose owder columns in the same way as on paper eets, the Rf values of the porphyrins cor- esponding with the Rf values of the same orphyrins in the paper sheet chromato- raphy.

EXPERIMENTAL

The porphyrins used were uroporphyrin I, oproporphyrin III and mesoporphyrin, hich have been generously supplied by prof. imington as the methyl esters.

The porphyrin esters were hydrolysed in e usual way with 7 N HCl for 32 hours, vaporated to dryness in a vacuum dessicator ver NaOH pellets, dissolved in small mounts of 30 % 10 % NH_3 in acetone. mall amounts of Wathman cellulose owder for chromatography were mixed ith the porphyrin solutions and dried a vacuum dessicator over night. The dry llulose powder which contained the por- nyrins, were bottled and kept in the dark as andard sources of analytical material.

The behavior of the porphyrins on small olumns of cellulose powder packed tightly ith a glasrod were tested using ascending romatography. It was found that the porphyrins behaved completely in the same way as on paper sheets. However, the ascending method was very inconvenient to work with as the elution of the porphyrins could be done only with difficulty, and the following procedure was worked out using descending development and elution of the different porphyrins by liquid chromatography.

Approximately 15 cm long columns and of varying diameter depending on the amount of porphyrin to be analysed were used.

A small stopper of glas wool was placed in the lower end of the column and Wathman cellulose powder for chromatography was packed into the column up to approximately 12 cm with a glasrod flatened in one end. The porphyrin containing cellulose sample was then added and packed in a narrow strip and finally an approximately 1 cm high layer of pure cellulose powder was packed on the top of the column to protect the porphyrin containing zone during the addition of the eluent.

A mixture of 2—6 lutidine/water 6/2 was added and allowed to kreep down the column. The porphyrins were separated during the passage of the solvent mixture down the column. By measuring the distance travelled by the front and by the different zones and calculating the Rf values we found the following approximate values: Mesoporphyrin 0.95, coproporphyrin 0.53, while uroporphyrin had not moved at all.

The speed of elution was increased after the development of the chromatogram on the column, by slight pressure. This brings out

the 2 carboxylic porphyrin. Then the water content of the water/lutidine mixture was increased from 2/6 to 4/6, and the 4 carboxylic porphyrin brought down. At the same time the uroporphyrin began to move. When all porphyrins but uroporphyrin were eluted from the column a drop of conc. NH_3 was added to 25 ml of the 6/4 lutidine/water mixture, and the uroporphyrin brought down rapidly and in a highly concentrated band.

The column must be kept in the dark during chromatography.

The different zones eluted were tested on paper sheets according to Eriksen (1953), and found to behave as 2, 4 and 8 carboxyli porphyrins respectively. Thus the separatio of the different porphyrins has been accom plished according to the same principal as i the paper sheet chromatography.

SUMMARY

A simple method for large scale separatio of porphyrin pigments according to th number of acidic groups has been describe

REFERENCES

Eriksen, Leif: Paper chromatography of por phyrin pigments. Scandinav. J. Clin. & La Investigation *5*, 155, 1953.

candinav. J. Clin. & Lab. Investigation 99—100, 9, 1957.

A NOTE ON THE DETERMINATION OF SERUM CHOLESTEROL BY THE LIEBERMANN-BURCHARD REACTION

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(Received for publication August 29, 1956)

As the determination of serum cholesterol s extensively used as a routine examination n most clinical laboratories there is a need or a method which is rapid and at the same me sufficiently exact and specific. In this aboratory we have found the method de-cribed by Pearson, Stern & McGavack 1952) to fulfill these conditions.

In this method the Liebermann-Burchard eaction (LBR) is carried out directly on erum. The serum lipids are not extracted or is the cholesterol precipitated as in most ther methods. The extinction of LBR color measured photometrically at the wave-ngth of 5500 Å, not at 6250 Å, where the olor has its maximum absorption and which recommended in most other methods. As ie authors do not explain this divergence ieir instruction has caused some confusion nd some errors in the way in which the ethod is carried out. In this paper it is emonstrated that the reason why the wave-ngth 5500 Å has to be used is the fact that ilirubin gives a reaction very similar to ie LBR.

It has repeatedly been observed that other ubstances than cholesterol may influence ie LBR. According to Sole (1947) one gets ie typical green color with, for example, ilirubin, hemoglobin and carotene. As the iolesterol determinations are of special im-ortance in jaundice the reaction with bili-ubin may involve considerable source of ror. This error is eliminated when the ex-nction at 5500 Å is used instead of 6250 Å, nd if the estimation is made against a blank onsisting of serum, correspondingly diluted

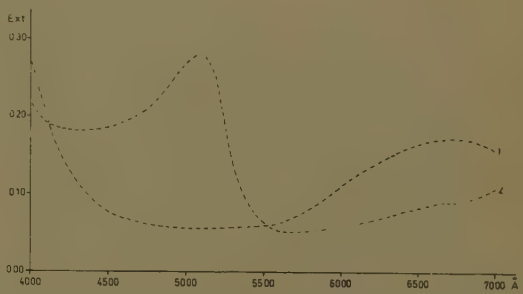

Fig. 1. Absorption curves of 1) the Liebermann-Burchard reaction carried out on a solution of bilirubin in chloroform (12.4 mg per 100 ml) and 2) of the same bilirubin solution correspondingly diluted with p-toluenesulfonic acid. — Ordinate: extinction coefficient, abscissa: wave-length.

with p-toluenesulfonic acid. This elimination of the error is due to the fact that at 5500 Å, but not at 6250 Å, the extinction of the color due to the presence of bilirubin in the LBR equals the extinction of the corresponding bilirubin blank.

This is demonstrated in Fig. 1. On a solution of bilirubin (Merck, puriss) in chloroform, containing 12.4 mg per 100 ml, the LBR was carried out as described by Pearson, Stern & McGavack (1952). Between the wave-lengths of 4000 and 7000 Å the extinctions of sample and blank were determined separately in a Beckmann B photometer. Both were read against water as blank.[1] The extinction curves cross each

[1] According to Jones & Moreland (1955) the mixture may explode when transferred to cuvettes if the latter contain water.

other at 5500 Å. In other experiments it was found that the LBR carried out on a pure cholesterol solution follows the Lambert-Beer law at 5500 Å as well as at 6250 Å.

In several sera total cholesterol was determined by both the method of Pearson *et al.* and the Bloor method as described by Bloor, Pelkan & Allen (1922). The results were in good agreement when the wave-length 5500 Å was used in the method of Pearson *et al.*

In three icteric sera the total cholesterol was determined according to the method of Pearson *et al.*, the Bloor method as described by Bloor, Pelkan & Allen and the digitonia precipitation method of Sperry & Schoenheimer (1934). The bilirubin was determined according to Jendrassik & Grof (1938). The results appear in Table I.

Table I. *Comparison of the values for concentration of total serum cholesterol when determined with the method of Pearson et al., the Bloor method and the method of Sperry & Schoenheimer (SS). All concentrations in mg per 100 ml serum.*

Serum no.	Bilirubin	Cholesterol		
		Pearson *et al.*	Bloor	SS.
1	22.3	630	615	600
2	16.8	560	570	540
3	5.4	600	590	595

CONCLUSION AND SUMMARY

The method of Pearson, Stern & McGavack (1952) for the determination of serum cholesterol is time-saving and gives results in good agreement with other methods. Bilirubin influences the Liebermann-Burchard reaction. This makes the cholesterol result erroneous if the extinction at the absorption maximum, 6250 Å, is used. The error is eliminated if the extinction at 5500 Å is used and if the extinction is read against a serum blank. This elimination of the error is due to the fact that at 5500 Å the extinction of the LBR-color wtih bilirubin equals that of the blank.

REFERENCES

Bloor, W. R., Pelkan, K. F. & Allen, D. M.: Determination of fatty acids and cholesterol in small amounts of blood plasma. J. Biol. Chem. *52,* 191, 1922.

Jendrassik, L. & Grof, P.: Vereinfachte photometrische Methoden zur Bestimmung des Bilirubins. Biochem. Z. *297,* 81, 1938.

Jones, B. J. & Moreland, F. B.: p-toluenesulfonic acid, cholesterol determination and explosions. Clin. Chem. *1,* 345, 1955.

Pearson, S., Stern, S. & McGavack, T.H.: A rapid accurate method for the determination of total cholesterol in serum. Anal. Chem. *25,* 813, 1953.

Schoenheimer, R. & Sperry, W. M.: A micromethod for the determination of free and combined cholesterol. J. Biol. Chem. *106,* 745, 1934.

Sols, A.: Determination directa de colesterina total en suere. Rev. espan. fisiol. *3,* 225, 1947.

ndinav. J. Clin. & Lab. Investigation 101—102, 9, 1957.

HEAT TOLERANT, LOW RESISTANCE BREATHING VALVE

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(Received for publication November 1, 1956)

For spirometry during exercise or hyper-ntilation, a leakproof breathing valve ich requires a minimum of opening pres-re and offers small resistance to large flows, is necessary to obtain true ven-tory values.

If such a valve is used by patients suffer-g from infectious lung diseases, it should eferably tolerate dry sterilization without terioration or distortion of any of its parts. In the commonly used types of breathing lves there are membranes of mica, flaps rubber, or plastic parts (Comroe, 1950) ich do not tolerate dry sterilization.

In the following a breathing valve, which think fulfills all the requirements men-ned, is described.

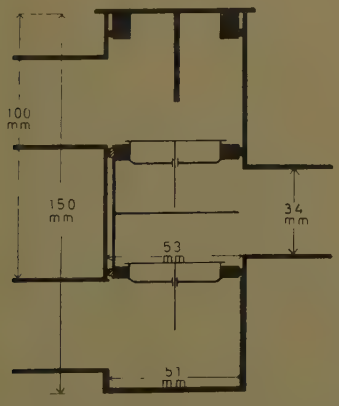


Fig. 1. Vertical section through the valve.

Fig. 2. Inner cylinder with valve seats and membranes.

DESCRIPTION OF THE VALVE

A brass pipe with an inner diameter of 51 mm and 150 mm long is used for the housing. Short pieces of brass pipe with an internal diameter of 34 mm are used for connection with the in- and expiratory tubes and the mouthpiece.[1]

The valve seats are brass rings with an outer diameter of 51 mm and an inner diameter of 34 mm.

[1] Mouthpieces with this internal diameter are obtainable from Mc Ewan and Smith, Doylestown, Pennsylvania, U. S. A.

Fig. 3. Assembled valve.

The outer edge of each ring has 2 mm deep groove for an 0-ring (Angus no. R 150). The inner edge has a 1 mm elevation which serves as a seat for the circular, 0.2 mm thick steel membrane with a diameter of 38 mm. The membranes are equipped with steering pins.

For constructional details, see Fig. 1.

The two valve seats are connected with a section of a cylinder, which facilitates assembling of the valve, a plate between the valve seats reduces dead space (Figs. 1 and 2).

The top of the housing is closed with a lid equipped with an 0-ring.

The assembled valve is shown in Fig. 3.

The valve has to be used in a fairly vertical position, or springs must be inserted.

VALVE CHARACTERISTICS

All air openings in the valve correspon to a tube with an inner diameter of 34 m

The pressure required to lift a thorough wet membrane from its seat is 0.5 mm water. A reduction of the top surface of t seat would diminish this opening pressur

The resistance to air flows of 50, 100, 2 and 300 l/min. is 0.5, 1, 2 and 3 mm of wat respectively.

This compares favorably with the sistance of the valve recently published McKerrow & Otis (1956), who with the valve found an expiratory resistance approximately 4.5 to 12.5 mm of water airflows of 50 to 300 l/min.

The 0-rings which have the least toleran to heat, can tolerate temperatures up 170° C.

REFERENCES

Comroe, J. H., Jr. & Kraffert, Nancy, H.: Valv stopcocks and tubing: sterilization procedur In: Methods in medical research II. The Ye Book Publishers, Inc. Chicago 1950, p. 79.

McKerrow, C. B. & Otis, A.: Low Resistan valve for hyperventilation. J. Appl. Physi *9*, 497, 1956.

Letters to the Editors

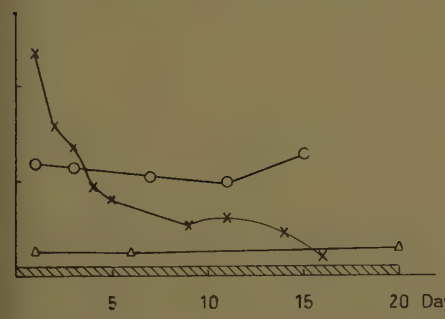
ndinav. J. Clin. & Lab. Investigation 103—104, 9, 1957.

A NEW SPECIFIC LIVER TEST. DETERMINATION OF RNITHINE CARBAMYL TRANSFERASE IN HUMAN SERUM

n the mammal, enzymic formation of urea m CO_2, NH_3 and ornithine takes place lusively in liver (Grisolia & Cohen, 1951). e step in urea synthesis is the formation citrulline (Jones, Spector & Lipmann, 5):

rbamylphosphate + ornithine ⟶ citrulline + phosphate

his enzymic reaction involves a carbamyl nsfer and the purified enzyme is highly cific for ornithine (Reichard, 1956). The ne ornithine carbamyl transferase (OCT) proposed and this enzyme was shown be identical with the enzyme discovered Krebs, Eggleston & Knivett (1955). This yme catalyzed the arsenolysis of citrul- to CO_2, NH_3 and ornithine. These authors also demonstrated that the arsenolysis of citrulline was exclusively localized in mammalian liver.



1. OCT-units the same as in the table. — ee typical curves illustrating variation of OCT level during disease.
x—x Hepatitis infectiosa.
o—o Cancer vesicae fellae cum metastasibus hepatis.
Δ—Δ Cirrhosis hepatis.
ded area corresponds to normal OCT level.

Group	No. of cases	OCT units range	means
Healthy persons	15	0—3	1,4
Ulcus duodeni ..... (2)			
Nephritis ac ..... (2)			
Pneumonia ac ..... (2)	6	1—4	2
Infarctus cord.	5	0—6	2
Cholelithiasis ..... (10)			
(without fever or pains)	9	0—6	3
	1	15	
Cholecystitis ..... (8)			
(with fever and pains)	6	12—88	38
	2	1—2	
Cirrhosis hepatis	3	9—15	13
Cancer cum metastasibus			
hepatis ..... (4)	3	20—100	
	1	6	
Cancer sine metastasibus			
hepatis	2	1—5	
Hepatitis infect. .. (16)	12	10—248	*
	4	5—9	*

* First 2 days after onset of icterus always above 100.

OCT-units here used are only relative figures referring to applied standard condition. — Cirrhosis hepatis and cancer verified by microscopic examination. Samples from patients with infarctus cordis taken between 12 and 36 hours after onset of illness.

Damage to cells containing glutamic oxaloacetic transaminase results in an increased level of this enzyme in serum (LaDue, Wróblewski & Karmen, 1954). It might likewise be assumed that damage to liver cells containing OCT results in an elevated OCT level in serum. The present communi-

cation describes the occurence and variation of OCT in normal and pathological sera.

The arsenolysis of citrulline was a convenient method for measuring OCT activity. With citrulline-carbamyl-^{14}C as substrate measurements of the formation of radioactive CO_2 allowed the quantitative determination of very small amounts of OCT.

For this purpose 0.5 ml of serum was incubated with 250 μ moles of sodium arsenate, pH 7.1, 2 μ moles of citrulline-carbamyl-^{14}C in an final volume of 1 ml. The formation of $^{14}CO_2$ under standard conditions was measured and was proportional to the content of OCT in serum.

For results see the accompanying table and figure. In the present material the OCT level was raised only in liver and bile diseases. Especially high values were found in infectious hepatitis at the onset of icterus with OCT values 200 times thet normal range. In acute cholecystitis strongly elevated values were also observed. Moderate i creases were found in liver cirrhosis. Ti curves of the OCT level may give valuab diagnostic and prognostic information.

These preliminary results indicate th measurement of the amount of OCT serum probably can serve as a specific liv test.

REFERENCES

Grisolia, S. & Cohen, P. P.: Study of citrulli synthesis with soluble enzyme preparatio J. Biol. Chem. *191*, 189, 1951.

Jones, M. T., Spector, L. & Lipmann, F.: Carb myl phosphate, the carbamyl donor in enz matic citrullin synthesis. J. Am. Chem. Soc. 819, 1955.

Krebs, H. A., Eggleston, L. V. & Knivett, V. A Arsenolysis and phosphorolysis of citrulline mammalian liver. Biochem. J. *59*, 185, 1955.

La Due, J. S., Wróblewski, F. & Karmen, A Serum glutamic oxalatic transminase activi in human acute transmural myocardial infar tion. Science *120*, 497, 1954.

Reichard, P.: Ornithine carbamyl transferase fro rat liver. Acta Chem. Scandinav. In press.

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ACETYLATION IN DIABETES MELLITUS

On the basis of their experiments on oxanized rats, Charalampous & Hegsted 949) suggested that a diminished acetyl-ion of, *e. g.*, foreign amines is present in abetes. The restoration of this defect to rmal with ATP and with intermediates of e citric acid cycle led the authors to assume at the effect was due to a deficient forma-n of high energy phosphate bonds in dia-tes. This view has been held up to the esent and the decreased acetylation is taken evidence for an impaired oxidative phos-orylation in the diabetic organism (Stadie, 55, 1956). In seven cases of human dia-tes *under insulin treatment* Gershberg & uhl (1950) found the acetylation rate to be rmal. Since our studies on acetylation of aminobenzoic acid (PABA) in diabetic man subjects have resulted in a quite op-site view, a short account of them is given re.

As a measure of the acetylation capacity e acetylated/total PABA ratio in a 3-hour inary sample collected after oral admini-ration of 1 gm PABA was determined Bratton & Marshall, 1939). The data ob-ined in 31 diabetics and in 22 normal sub-cts are presented in Fig. 1. The groups ere matched as to age, sex and body build. The diabetic subjects are divided into three bgroups according to the stage and the sponse to treatment of their disease. The ses included in the first column had a more less severe ketosis and some, but not all, them received insulin when the acetylation t was made. The patients placed in the second column were all under insulin treatment and had a variable degree of hyperglycemia and glucosuria but no signs of ketosis. Their diabetes was not controllable with oral antidiabetics or mere diet. The members of the third group received no treatment at the time when tested for acetylation but later they all revealed a favourable response to BZ 55 or dietary regimen.

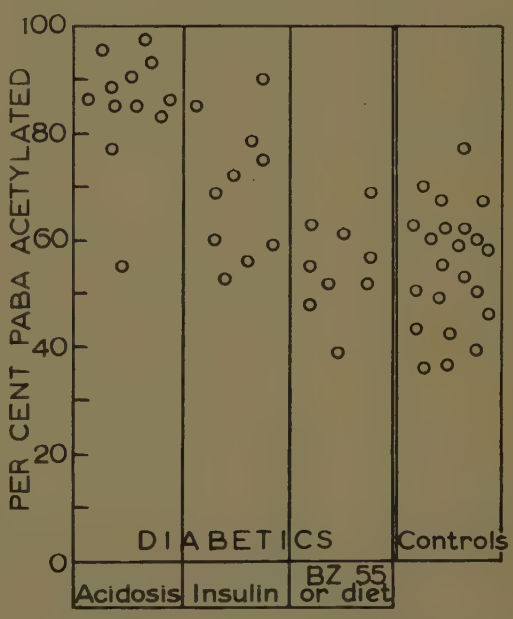

Fig. 1. Percentage ratio of acetylated/total PABA excreted in urine during 3 hours after oral ingestion of 1 gm by diabetic and normal human subjects. For explanation of the grouping see text.

It is obvious that the ability to acetylate a foreign amine such as PABA is definitely increased during diabetic acidosis and often over normal limits also in insulin-needing diabetics without acidosis, but it is normal in cases which are later controlled by oral antidiabetics or diet only. No distinct correlation exists between the acetylation percentage and the fasting level of blood sugar. In previously untreated cases with acidosis, the acetylation percentage falls somewhat on insulin administration but often remains high in spite of an apparently perfect control of the diabetic state.

The exact interpretation of these findings by means of the present knowledge of the biochemistry of human diabetes is not fully clear. Probably the increased acetylation of a foreign substance reflects the same metabolic fault as increased acetoacetate synthesis. The observation need not necessarily, however, be incompatible with the decreased oxidative phosphorylation in diabetes sinc it is possible that an accelerated acetylatio reaction of this kind can proceed *in viv* without a normal formation of energy-ric phosphate compounds. The discrepancy be tween the results of acetylation studies i alloxan-induced rat diabetes and the spon taneous human disease points to a differenc between these two diabetic states.

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December 3, 1956.

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W. Kerppola.

REFERENCES

Bratton, A. C. & Marshall, E. K. Jr.: A ne coupling component for sulfanilamide dete mination. J. Biol. Chem. *128,* 537, 1939.

Charalampous, F. C. & Hegsted, D. M.: Acety ation in the diabetic rat. J. Biol. Chem. *18* 623, 1949.

Gershberg, H. & Kuhl, W. J. Jr.: Acetylatio studies in human subjects with metabolic di orders. J. Clin. Investigation *29,* 1625, 1950.

Stadie, W. C.: Current views on the mechanis of insulin action. Am. J. Med. *19,* 257, 1955.

— Recent advances in insulin research. The Ban ing Memorial Lecture 1956. Diabetes *5,* 26 1956.

candinav. J. Clin. & Lab. Investigation 107—108, 9, 1957.

THE MECHANISM OF CREATINURIA DURING IMMOBILIZATION IN BED

In investigations on humans, Sandberg, Iecht & Tyler (1953) have confirmed earlier nimal experiments showing that creatine is ynthesized in the liver. Benedict, Kalinsky, carrone, Wertheim & Stetten (1955) have ed N^{15}-glycine, a precurser of creatine, to atients with severe progressive muscular ystrophy and thereby presented evidence nat the creatinuria in this disease is caused y a failure of newly formed creatine to nter the muscle. The finding of a urinary reatine with an initial concentration of N^{15} ar higher than that found in the creatinine xcreted in the same period was used as the ain basis for this conclusion.

Heilskov & Schønheyder (1955) found a pronounced creatinuria as well as a negative nitrogen balance in normal persons immobilized in bed. These persons had a normal diet containing about 125 g meat per day. A similar creatinuria has now also been found in a normal person kept on a creatine-free diet, cf. Table I.

A normal man (FS), aged 21, was immobilized in bed for two weeks on a creatine-free diet. Before immobilization the urinary excretion of creatinine varied between 1620 and 1940 mg per 24 hours, and the urine was practically free of creatine. The day before immobilization the concentration of creatinine and creatine in serum was 0.90 mg and 0.53 mg per 100 ml respectively. The determinations of

Table I. *Creatine and creatinine in serum and urine from subject FS.*

Day of imm.	Urinary creatinine. mg excreted	Urinary creatine. mg excreted	Serum creatinine. mg/100 ml	Serum creatine. mg/100 ml
0[1]			0.90	0.53
1	1914	34	0.92	0.48
2	1702	167	0.81	0.75
3	1680	200	0.81	0.83
4	1676	134	0.77	0.75
5	1810	177		
6	1869	247	0.92	0.89
7	1729	404	0.83	0.92
8	1693	343	0.80	0.87
9	1651	371	0.67	0.82
10	1569	408		
11	1640	322	0.76	0.92
12	1606	263	0.64	1.03
13	1743	203	0.76	0.89
14	1608	151	0.74	0.90

[1] Day 0 indicates the day before immobilization.

Table II. *N^{15} atom per cent excess in urinary creatinine and creatine after feeding N^{15}-glycine.*

Date	Hours after feeding N^{15}	Urinary creatinine. Atom per cent excess	Urinary creatine. Atom per cent excess
1.21. 8 A.M.—2 P.M.	3	.0144	.1445
1.21. 2 P.M.—1.22. 8 A.M.	15	.0145	.1055
1.22. 8 A.M.—1.23. 8 A.M.	36	.0108	.0677
1.23. 8 A.M.—1.24. 8 A.M.	60	.0102	.0233
1.24. 8 A.M.—1.25. 8 A.M.	84	.0045	.0135

creatine and creatinine were carried out according to the method of Borsook (1935). During the whole immobilization period the nitrogen balance was negative with a maximum loss of 6.7 g on the 8th day.

On the 11th day of immobilization at 8 a.m. 1 g of N^{15}-glycine (34 atom per cent excess) was fed, and during the following days the exreted amounts of creatine and creatinine were isolated as creatinine zinc chloride from a series of urine portions, a modification of the method of Bloch & Schoenheimer (1939) being used. The crystallized samples were subjected to wet ashing with sulphuric acid, and the N^{15} atom per cent excess was measured in a Metropolitan Vickers MS III mass spectrometer.

It is seen (Table II) that the contents of N^{15} are far greater in creatine than in creatinine during the first days after the administration of N^{15}-glycine.

The results arrived at do not support the view that the excreted creatine should originate from a surplus of creatine leaving the muscle cells, but they do favor the conception that the increased amount of creatine in blood and urine is caused by a diminished uptake in the muscles of the endogenously produced creatine. This decrease in utilization of creatine is in accord with the finding of a diminished utilization of dietary protein during immobilizing bed rest (Schønheyder, Heilskov & Olesen, 1954).

REFERENCES

Benedict, J. D., Kalinsky, H. J., Scarrone A. Wertheim, A. R. & Stetten, Jr., DeWi. The origin of urinary creatine in progressive muscular dystrophy. J. Clin. Investigation *34*, 141 1955.

Bloch, K. & Schoenheimer, R.: Studies in protein metabolism XI. The metabolic relation of creatine and creatinine studied with isotopic nitrogen J. Biol. Chem. *131*, 111, 1939.

Borsook, H.: Micromethods for determination of ammonia, urea, total nitrogen, uric acid, creatinine (and creatine), and allantoin. J. Biol Chem. *110*, 481, 1935.

Heilskov, N. C. S. & Schønheyder, F.: Creatinuria due to immobilization in bed. Acta Med. Scandinav. *151*, 51, 1955.

Schønheyder, F., Heilskov, N. C. S. & Olesen, K. Studies in disorders of muscle. X. The site of creatine synthesis in the human. Metabolism *2*, *22*, 1953.

Schønheyder, F., Heilskov, N. S. C. & Olesen, K. Isotopic studies on the mechanism of negative nitrogen balance produced by immobilization Scandinav. J. Clin. & Lab. Investigation. *6*, 178 1954.

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December 7, 1956.

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